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(54) Title: METHODS AND COMPOSITIONS FOR THE MANUFACTURE OF REPLICATION INCOMPETENT ADENOVIRUS

(57) Abstract: The present invention relates to complementing cell lines for the production of replication incompetent viruses, which significantly reduce or eliminate the presence of replication competent viruses. Methods to make and use the complementing cell lines are also provided, as are nucleic acid molecules, polynucleotides, and vectors for making the cell lines. In particular, the present invention relates to complementing cell lines for the production of replication incompetent adenoviruses (Ad), which significantly reduce or eliminate the presence of replication competent Ad (RCA) and can serve for the large scale production of infectious replication incompetent adenovirus particles that may be used for the treatment of human patients as for example in gene therapy. The present invention further relates to an assay for detecting the presence of replication competent virus particles, in particular RCA, in a stock of infectious replication incompetent virus particles, in particular replication incompetent adenovirus particles, which employs a real time quantitative PCR assay with a sensitivity level to detect one replication competent virus particle per $\geq 10^9$ replication incompetent virus particles.

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METHODS AND COMPOSITIONS FOR THE MANUFACTURE OF REPLICATION INCOMPETENT ADENOVIRUS

Background of the Invention

Field of the Invention

The present invention relates to complementing cell lines for the production of replication incompetent viruses, which significantly reduce or eliminate the presence of replication competent viruses. In particular, the present invention relates to complementing cell lines for the production of replication incompetent adenoviruses (Ad), which significantly reduce or eliminate the presence of replication competent Ad (RCA) and can serve for the large scale production of infectious replication incompetent adenovirus particles that may be used for the treatment of human patients as, for example, in gene therapy. As well the invention relates to a method for the large scale production of infectious recombinant replication incompetent virus particles, in particular replication incompetent adenovirus particles, harboring an exogenous sequence of interest and to a stock of infectious replication incompetent virus particles, in particular replication incompetent adenovirus particles, which is free from replication competent virus particles, in particular RCA. The invention further relates to nucleic acid complementation elements and to recombinant vectors comprising such nucleic acid molecules for transfecting a eukaryotic cell line which significantly reduce the presence of RCA and to a method therefor. In addition, the present invention relates to an assay for detecting the presence of replication competent virus particles, in particular RCA, in a stock of infectious replication incompetent virus particles, in particular replication incompetent adenovirus particles, which employs a real time quantitative PCR assay with a sensitivity level to detect one replication competent virus particle per $\geq 10^9$ replication

incompetent virus particles. The invention further relates to a method of detecting the presence of replication competent virus particles, in particular RCA, in a stock of infectious replication incompetent virus particles, involving utilization of such an assay, and a kit which supplies the components necessary to perform the assay.

Related Art

Therapeutic strategies in various disease states include nonspecific measures to mitigate or eliminate a cell dysfunction and prevent cell death, replacement of a missing or malfunctioning protein, introduction of functional nucleic acids (RNA or DNA) into cells to replace a mutated gene and introduction of novel genetic constructs to alter a cellular function. Advances in recombinant DNA technology have had a major impact on each of these therapeutic possibilities and nucleic acid transfer appears to be a promising modality.

Viral vectors permit the expression of exogenous genes in eukaryotic cells, and thereby enable the production of proteins which may require post-translational modifications unique to animal cells. Gene therapy vectors derived from viruses require various modifications to eliminate their disease-causing potential, yet retain their ability to: 1) replicate under controlled conditions for preparation of viral stocks during manufacturing; and 2) to infect and deliver the desired therapeutic gene to the diseased cell. Elimination of the disease-causing potential of viral vectors is normally achieved by deleting a subset of genetic elements from the viral genome to prevent independent viral replication in patients. To manufacture these vectors, they are commonly propagated in producer cells (or packaging cells) engineered to complement the replication incompetent virus by expressing the subset of genetic elements deleted from the viral genome. A number of animal viruses have been employed as viral vectors, including, adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, herpesviruses, poxviruses, alphaviruses, and picornaviruses.

The following characteristics of adenovirus (Ad) make it an attractive tool for gene transfer or immunization: (1) the structure of the adenovirus genome is well characterized; (2) large portions of viral DNA can be substituted by foreign sequences; (3) the recombinant variants are relatively stable; (4) the recombinant virus can be grown at high titer; (5) no known human malignancy is associated with adenovirus; and (6) the use of attenuated wild-type adenovirus as a vaccine is safe.

Generally, such replication-incompetent Ad vectors are constructed by inserting the gene of interest in place of, or in the middle of, essential viral sequences such as those found at the E1 locus (Berkner, *BioTechniques 6*:616-629 (1988); Graham *et al.*, *Methods in Molecular Biology, 7*:109-128, Ed: Murcy, The Human Press Inc. (1991)). This deletion or insertional inactivation of essential viral genes results in crippling the ability of Ad to replicate, hence the term replication-incompetent Ad. In order to propagate such vectors in cell culture they must be provided with the deleted element, (*e.g.*, the E1 proteins in the case of an E1 deleted vector).

The elucidation of the nucleotide sequence of many Ad subtypes has enabled a precise characterization of the genomic organization thereof. The nucleotide sequence of human adenovirus type 5 (Ad5) is available from GenBank under accession number M73260. In simplistic terms the adenovirus genome comprises: (1) two inverted terminal repeats (ITRs) at each end (5' and 3') which are essential for viral replication; (2) the early region 1 (E1) containing the E1A and E1B regions, both indispensible for replication, and polypeptide IX (pIX) which is essential for packaging of full-length viral DNA and forms a component of the viral capsid; (3) the E2, E3 and E4 regions, with E3 being dispensable for replication (reviewed in Acsadi *et al.*, *J. Mol. Med. 73*:165-180 (1995)); and (4) the late regions L1 through L5 which mainly encode virion proteins and are all dispensible for replication (*The Adenoviruses*, Ed. Harold Ginsberg, 1984, Plenum Press, NY).

Human Ad serotypes 2 and 5 have been used as vectors for efficient introduction of genes into several cell types both *in vitro* and *in vivo* (reviewed in Trapnell *et al.*, *Current Opinion Biotech. 5*: 617-625 (1994); and Acsadi *et al.*, *J. Mol. Med. 73*:165-180 (1995)). Several factors need to be taken into consideration during the generation of Ad recombinants, among which is the impaired growth characteristics of some of the recombinants (Imler *et al.*, *Gene Ther. 2*:263-268 (1995); Massie *et al.*, *BioTechnol. 13*:602-608 (1995); and Schaack *et al.*, *J. Virol. 69*: 3920-3923 (1995)). These characteristics complicate the screening, propagation and production of high quality recombinant viral stocks with high titers (more than 10^{11} pfu/ml). Critical issues relating to the characterization of such Ad vectors for gene therapy have been reviewed in relation to clinical trials of the cystic fibrosis gene therapy (Engelhardt *et al.*, *Nature Genetics 4*:27-34 (1993); Zabner *et al.*, *Cell 75*:207-216 (1993); Boucher *et al.*, *Hum. Gene Ther. 5*:615-639 (1994); Mittereder *et al.*, *Hum. Gene Ther. 5*:717-729 (1994); and Wilmot *et al.*, *Human Gene Ther. 7*:301-318 (1996)). Potential sites for the insertion of a gene of interest in the recombinant Ad vectors comprise the E1 region, the E4 region, the E1 and E3 regions (*i.e.* E1/E3-deleted Ad recombinants), the E1 and E4 regions, or the region between the end of the E4 and the beginning of the 3' ITR sequences.

As alluded to above, E3-deleted recombinants are replication competent. E1-and/or E4-deleted recombinants, however, are unable to replicate and the missing gene products are provided *in trans* by an E1-complementing cell line such as 293 (Graham *et al.*, *J. Gen. Virol. 36*:59-72 (1977); Lochmuller *et al.*, *Hum. Gene Ther. 5*:1485-1491 (1994)) or 911 (Fallaux *et al.*, *Hum. Gene Ther. 7*: 215-222 (1996)), and E4-complementing cell line such as W162 (Weinberg, D., and Ketner, G., *Proc. Natl. Acad. Sci. USA 80*:5383-5386 (1983)), or an E1/E4 complementing cell line such as IGRP2 (Yeh, P., *et al.*, *J. Virol. 70*:559-565 (1996)). The 293 cells were established in the 1970s by stable transfection of diploid human embryonic kidney cells with sheared human Ad5 DNA. The cell line was originally constructed in the course of a study on the transforming

potential of the E1 genes of Ad. Recently, Graham and colleagues mapped the cellular-Ad5 DNA junctions in the chromosome of 293 cells and showed the presence of contiguous Ad5 sequences from the left end of the Ad5 genome to nucleotide (nt) position 4137 (Louis *et al.*, *Virology 233*: 423-429 (1997)). A second E1 complementing cell line, 911, was derived from diploid human embryonic retinoblast (HER) cells and harbors nt 80-5788 of the human Ad5 genome (Fallaux *et al.*, *Hum. Gene Ther. 7*: 215-222 (1996); also described in WO 97/00326, published January 3, 1997; ECACC No. 95062101).

The maximum deletion of approximately 3.0 kb in the E1 region of E1-deleted Ad vectors leaves intact the ITR sequence, the packaging signal at the left end of the adenovirus DNA (nt 188-358) and the pIX coding region (starting at nt 3511). The E1 deletion, combined with a useful secondary deletion of a 1.9 kb Xba I fragment (79 and 85 mu) in the nonessential E3 gene, allows for inserting approximately 7 kb of foreign DNA sequences in this first-generation recombinant. Extensions of the deletion in the E3 regions further increase the insert capacity to 8 kb, which meets the size requirements for most of the gene therapeutics (Bett *et al.*, *Proc. Natl. Acad. Sci. 91*:8802-8806 (1994)).

Unfortunately, it has been documented that replication competent adenovirus (RCA), also termed "revertant" adenovirus, can spontaneously appear during infection and replication of the E1- or E1/E3-deleted recombinant Ad in 293 or 911 cells. RCA is produced in the E1 complementing cells through the acquisition of the E1 region ("complementation element") contained in the cells by the process of homologous recombination. RCA is formed at a very low frequency, but the E1-positive revertants seem to have a growth advantage with respect to their E1-negative counterparts. The newly generated replication-competent Ad eventually outgrows the original replication-deficient Ad in large scale preparations (Lochmuller *et al.*, *Hum. Gene Ther. 5*: 1485-1491 (1994)). The presence of these revertants could thus jeopardize the safety of human gene therapy trials, especially when one considers the number of infectious viral particles required in certain applications.

Experiments performed with mouse muscle have taught the use of $2x10^9$ virus particles to transduce more than 80% of the muscle fibers. Since a human muscle is approximately 2500 times larger, that would translate the use of approximately 10^{12}-10^{13} viral particles to inject a single human muscle in order to achieve similar transduction rates. Assuming the presence of as little as 1/10 particles of $E1^+$ revertant RCA in the stock, 10^{11} replication-competent viral particles would be injected into the patient's muscle tissue. It is clear that such an approach would fail to satisfy regulatory agencies.

The presence of homologous nucleotide sequences in the Ad vector overlapping the same sequences in the complementation element is responsible for homologous recombination and subsequent RCA production. 293 cells have significant overlapping homology with most, if not all, currently available E1 deleted Ad vectors. Indeed, 293 cells have been deemed not suitable for large scale production of clinical grade material since batches are frequently contaminated with unacceptably high levels of replication competent adenovirus (RCA) arising through recombination (Imler *et al.*, *Gene Ther. 3*: 75-84 (1994)). It should be stressed that the same authors have reported failure of numerous attempts to construct stable and efficient E1-complementing cell lines, confirming that such an endeavor is therefore not a trivial task.

In an attempt to solve the problem of RCA generation Imler *et al.* produced an E1-complementing cell line by stably transforming human lung A549 cells with E1 sequences containing the E1a, E1b and pIX regions (Imler *et al.*, 1996, Gene Ther. 3: 75-84). A549 E1-complimenting cell lines were obtained which express high levels of E1a RNA and protein. Strikingly, however, the authors were unable to detect E1b protein expression in any of the A549 clones analyzed regardless of high level E1b RNA production from the clones. It is also reported therein that the A549 clones, testing positive for infection with E1-deleted Ad vectors, showed a transformed phenotype and that the amplification yields therewith were significantly lower than those obtained with 293 cells. Furthermore, the constructs used by Imler *et al.* in this work

contained a significant overlap of approximately 700 bp between the complementing element and the defective adenovirus vector at the 3' end of the E1 region. It follows that this overlap significantly increases the probability of homologous recombination and hence of the production of $E1^+$ revertant RCA. A disclosure of defective Ad vectors for the expression of exogenous nucleotide sequences in a host cell or organism, as well as vectors for the construction of E1-complementing cell lines, along the same lines is also found in Imler *et al.*, WO94/28152. However, this document fails to give an assessment of the yield of production of recombinant Ad by the complementing cell line, of the expression of the different adenovirus transcripts and proteins by the complementing cell line, and very importantly of the presence or absence of RCA during the production process leading to the generation of a stock of defective Ad harboring the exogenous sequence of interest. It should be noted that WO94/28152 claims to diminish the problem of RCA production by deleting the 5' ITR (a non-substantiated declaration).

An alternative system for reducing the risk of RCA formation during production of E1-deleted Ad vectors is described in Massie, US 5,891,690. The patent discloses an E1-complementing cell line having a stably integrated E1 complementation element comprising a portion of the Ad5 E1 region covering the E1a gene and the E1b gene under the control of the human beta actin promoter, and lacking the 5' ITR, the packaging sequence, and the E1a promoter. A specific cell line described and claimed by Massie, designated BMAdE1-220-8 (ATCC accession number CRL-12407) contains nt 532 - 3525 of Ad5, which includes E1a, the E1b promoter, and a portion of the E1b gene. However, the E1 deleted Ad disclosed in the patent contains a deletion of Ad5 nt 455-3333 and thus preserves a sequence overlap of 192 bp at the 3' end of the E1 complementation element. Furthermore, the complementation element in this cell line does not encode the 3' end of the E1b gene, coding for the 3' exon of the E1b 8.3 kDa polypeptide. The BMAdE1-220-8 cell and related lines described by Massie produce titers of E1 deleted Ad similar to those obtained on 293 cells. However,

no assessment of RCA generation on the claimed cell line is disclosed in the patent, and the presence of 3' overlap between vector DNA and the complementation element in the cell line described by the patent would suggest that RCA generation still remains a possibility.

An E1-packaging cell line termed "PER.C6" (ECACC NO. 96022940) has been described in the art (WO 97/00326, published on January 3, 1997; Fallaux *et al. Hum. Gene Ther. 9*: 1909-1917 (1998)). The PER.C6 and related cell lines described in the WO 97/00326 publication were derived from diploid HER cells stably transfected with a plasmid construct containing nt 459 - 3510 of human Ad5, corresponding to coding sequences for E1a and E1b, under the control of human phosphoglycerate kinase promoter. The cell line expresses Ad5 E1a and the two large E1b proteins, but it does not express the complete E1b 8.3 kDa protein as the second exon of the mRNA encoding this protein is transcribed from Ad5 nt 3595-3609. Prevention of RCA formation by the use of this cell line during propagation of E1-deleted Ad vectors requires the use of matching E1-deleted vectors which lack Ad5 nucleotides 459-3510, and such vectors are disclosed in the WO 97/00326 application. Unfortunately, however, as existing E1-deleted Ad vectors undergoing clinical testing contain various 5' and 3' boundaries at their E1 deletion, it would be time consuming and expensive to reengineer and reproduce safety and efficacy studies on each of the many therapeutic adenovirus-based medicines currently making their way through the regulatory approval process.

Compounding the problem of RCA generation is the lack of a reliable assay to screen large-scale rAd virus preparations for the presence of RCA. The current assay is a cell-based biological assay (Hehir, K., *et al.*, *J. Virol. 70*:8459-8467 (1996)). This assay has a limit of detection of 1 RCA per 10^9 rAd pfu. Clinical doses for rAd are in the range of $10^{12} - 10^{13}$ vector particles (~10^{10}-10^{11} rAd pfu), and takes 6 weeks to complete, making the current assay unworkable for routine quality control (QC) and product release testing due to the time, and the excessively large number of cells that would be required at this level. The

assay is limited by the cellular toxicity associated with the virion particle. The maximum MOI tolerated by non-E1 complementing cells (such as A549) is 10 pfu/cell, so very large numbers of cells are required to test for the presence of RCA. While it is theoretically possible to increase the number of cells to accommodate testing for RCA at higher sensitivity, this approach becomes impractical. Testing a single proposed dose of therapeutic product (10^{13} particles, approximately $2x10^{11}$ pfu) using this approach would require 2×10^{10} cells, corresponding to approximately 1000 (175 cm^2) flasks, including controls.

RCA derived from E1-deleted rAd obtain their E1 gene from the helper cells. Therefore the presence of E1-coding sequences in the viral DNA isolated from purified preparations of rAd can be a measure for the presence of RCA in the preparation. Polymerase chain reactions have the potential to detect these sequences rapidly and reproducibly with high sensitivity. There exists a small amount of literature describing attempts at developing conventional gel-based PCR RCA assays (Zhang, W., *et al.*, *Biotechniques 18*:444-447 (1995); Dion, L., *et al.*, *J.Virol.Meth. 56*:99-107 (1996)). Each of these reports suffers from a lack of sensitivity in the PCR analysis, with limits of sensitivity somewhere between 5,000 and 5,000,000 copies of the E1 target. In fact, the primer pair that had a sensitivity of 50,000 copies of target was demonstrated to detect one pfu of wild-type Ad virus in 10^9 pfu of E1-deleted virus (Zhang, *et al.*, *ibid.*).

Thus, there exists a need in the art for innovative approaches to large scale production of existing adenovirus vectors, and for the creation of alternative complementing cell lines that enable efficient production of safe, high quality, high titer adenovirus vectors without expensive and time consuming efforts directed at reengineering of the vectors themselves. Similarly, there exists a need in the art for a rapid, sensitive, and reproducible assay to detect RCA in high-titer rAd virus preparations.

Summary of the Invention

The present invention provides an improvement to currently available complementing elements and cell lines in that it provides stable complementing cell lines which encode and express adenovirus gene products, but which do not contain overlapping homology with adenovirus vectors. These complementation elements and cell lines are flexible in that they do not require the use of a limited subset of matched vectors in order to avoid RCA formation.

In one embodiment the present invention provides an isolated nucleic acid molecule comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, wherein the sequence of the polynucleotide is not a naturally occurring adenovirus nucleotide sequence.

In another embodiment the present invention provides an isolated nucleic acid molecule comprising a polynucleotide encoding at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide wherein the sequence of the polynucleotide is less than 97%, but at least 60%, identical to a naturally occurring adenovirus nucleotide sequence.

In yet another embodiment the present invention provides an isolated nucleic acid molecule comprising a polynucleotide encoding at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide wherein the polynucleotide will not hybridize to a naturally occurring adenovirus genome.

Other embodiments of the invention provide a complementation element comprising a nucleic acid molecule of the invention, a vector comprising a nucleic acid molecule of the invention, the use of a vector of the invention to generate a complementing cell line, and a complementing cell line stably transfected with a nucleic acid molecule of the invention.

In still another embodiment the invention provides a system for producing adenovirus vectors, comprising: (a) a complementing cell of the invention; and (b) an adenovirus vector having a nucleotide sequence which is not homologous to the complementation element, but in certain embodiments, would be

homologous to at least a portion of the complementing element contained in the cell but for the degeneracy of the genetic code.

It is another object of the invention to provide a method for producing RCA-free stocks of recombinant adenovirus vectors.

In yet another object of the invention is provided an assay for detecting the presence of replication competent virus, in particular, RCA, in a production stock of replication incompetent virus, particularly replication incompetent adenovirus, comprising: (a) subjecting a sample of the production stock to polymerase chain reaction (PCR) amplification to amplify a region of the genome of the replication competent virus which is deleted from the genome of the replication incompetent virus, thus forming a replication competent virus-specific double-stranded amplicon if such replication competent virus is present; (b) allowing the PCR reaction sample to hybridize with a signaling hybridization probe complementary to the replication competent virus-specific amplicon, thereby emitting a signal which is detectable only upon hybridization of the signaling hybridization probe to the replication competent virus-specific amplicon, or the genome of said replication competent virus; and (c) detecting the presence or absence of the signal. Other aspects of the assay include control PCR reactions which allow determination, in real time, of the relative quantities of replication competent virus and replication incompetent virus present in the production stock.

The invention further relates to a method of detecting the presence of replication competent virus in a production stock of replication incompetent virus, comprising the utilization of the above assay, and a kit which provides instructions, PCR primers and signaling probes for performing the assay.

Other aspects and advantages of the invention will be readily apparent from the following detailed description of the invention.

Brief Description of the Drawings

Figure 1 is a schematic diagram of the modified E1 complementation element generated by the method of Example 1. The first line (Wt Ad5) shows the location of the E1 region in wild-type (wt) adenovirus type-5 (Ad5) DNA. The second line (Wt E1 region) is an enlargement of the wild-type E1 and pIX genes, showing the nt numbers corresponding to the translational start and stop of the E1a and pIX proteins, respectively. The third line is a schematic of the modified E1 complementation element aligned with wild-type E1 in line 2. Heterologous constitutive promoter (*e.g.* PGK) is shown as "prm" and the polyadenylation signal (*e.g.* from bovine growth hormone or SV40) is shown as "pA". The darkened region between nt 3311 and 3609 at the 3' end of the E1b coding sequences in the complementation element represents the silent mutations designed to prevent homologous recombination between E1 sequences in the vector and E1 sequences in the complementing cell line. The fourth line shows a schematic representation of an E1-deleted Ad5 vector expressing human β-interferon as an example. As can be seen from the schematic, this vector, which is currently undergoing safety testing in human patients suffering from cancer, contains a large stretch of sequences from the 3' portion of the E1b gene.

Figure 2 shows the nucleotide sequence from nt 3309-3614 of the complementation element constructed according to Example 1 and Example 7. The wild-type Ad5 DNA sequence is shown in codon triplets. Single letter designation for amino acids is shown immediately above the first nt of each wild-type codon. Silent nucleotide substitutions introduced into the complementation element pQBI-pgk-E1.1 according to Example 1(A) and Example 7 are shown as italicized letters immediately below the nucleotide replaced in the wild-type Ad5 sequence (this complementation element includes all the substitutions shown below the wild-type sequence, including those that are underlined, and those that are boxed). Silent nucleotide substitutions introduced into the complementation element pQBI-pgk-E1.2 according to Example 1(B) and Example 7 are shown as

boxed italicized letters immediately below the nucleotide replaced in the wild-type Ad5 sequence from nucleotides. Silent nucleotide substitutions introduced into the complementation element pQBI-pgk-E1.3 according to Example 1(C) and Example 7 are shown as underlined italicized letters immediately below the nucleotide replaced in the wild-type Ad5 sequence from nucleotides. The boundaries of the wild type E1b intron are shown as downward arrows. The arrow marked SD is the splice donor site and the arrow marked SA is the splice acceptor site. The bold face letters within the intron represent the TATA box within the pIX promoter. The italicized sequence below the intron is the sequence of the SV40 late region intron used to replace the wild-type E1b intron in each of the three complementation elements, as described in Example 1 and Example 7. Asterisks represent the translational stop codons of either the 55- or 8.3 kDa E1b proteins. BglII refers to a restriction site in wild-type Ad5.

Detailed Description of the Invention

The present invention provides a method and system of producing replication-incompetent virus vectors which are free of replication competent virus, as well as nucleic acid molecules, complementation elements, vectors, and complementing cell lines useful in this method. In preferred embodiments, the present invention provides a method and system of producing replication-incompetent Ad in the absence of RCA, as well as nucleic acid molecules, complementation elements, vectors, and complementing cell lines useful in this method. Further, the present invention provides a method for screening large-scale replication-incompetent virus preparations, preferably rAd preparations, for the presence of replication-competent virus particles. The preferred Ad vectors are particularly well suited for use in delivering genes to a mammal, because these Ad vector preparations are free of contaminating RCA. Furthermore, the preferred Ad-complementing cell lines of the present invention are particularly well suited for production of Ad for preclinical and clinical use, as they are

readily adapted to growth in adherent or suspension cultures, as well as growth in serum free media using techniques well known to those of skill in the art. The serum-free-media adapted complementing cell lines harboring the constructs described herein are encompassed by the present invention.

The present invention accomplishes large scale production of RCA-free stocks of adenovirus vectors, as well as replication competent virus-free stocks of a variety of viral vectors through a novel strategy for the elimination of sequence homology between a complementation element contained in the production cell and the adenovirus vector. The strategy takes advantage of the degeneracy in the genetic code allowing for the creation of novel nucleic acid molecules which encode wild-type viral proteins yet lack sufficient sequence homology with the corresponding wild-type viral nucleic acid sequences to allow for homologous recombination leading to replication-competent virus production.

Definitions and General Techniques

Unless otherwise defined, all technical and scientific terms used herein have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA technology, genetics, virology and immunology. See, *e.g.*, Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (which is incorporated herein by reference in its entirety).

A nucleic acid molecule "comprising" a polynucleotide means that the nucleic acid molecule includes the polynucleotide but may also include other polynucleotides, *e.g.*, a nucleic acid molecule comprising a polynucleotide which encodes insulin may have additional polynucleotides 5', 3' or interspersed between those which are necessary to encode insulin such as an expression

control region, an untranslated region(s), an intron, and/or a polynucleotide which encodes a fusion protein.

A "transgene" is a nucleic acid molecule that is to be delivered or transferred to a mammalian cell. A transgene may encode a protein, peptide or polypeptide that is useful as a marker, reporter or therapeutic molecule. The transgene may also encode a protein, polypeptide or peptide that is useful for protein production, diagnostic assays or for any transient or stable gene transfer *in vitro* or *in vivo*. Alternatively, a transgene may not encode a protein but rather be used as a sense or antisense molecule, ribozyme or other regulatory nucleic acid molecule used to modulate replication, transcription or translation of a nucleic acid molecule to which it is complementary or to target a complementary mRNA for degradation.

"Expression control sequences" are polynucleotide regions that regulate the expression of a gene by being operably associated with the gene of interest.

"Operably associated" polynucleotides include both expression control sequences that are contiguous, *i.e.*, act in *cis*, with the gene of interest and expression control sequences that act in *trans* or at a distance, to control the gene of interest. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance protein stability; and when desired, sequences that enhance protein secretion.

A "transgene cassette" is a nucleic acid molecule comprising a transgene operably associated with expression control sequences.

A "virus genome" is the nucleic acid molecule backbone of a virus particle. The virus genome may contain point mutations, deletions or insertions of nucleotides. The virus genome may further comprise a foreign gene. A "native" or "naturally-occurring" virus genome is one which is isolated from a wild type virus growing in a natural animal host. Although a "naturally occurring" virus genome may be kept and propagated in a laboratory, it has not

been intentionally manipulated by, *e.g.*, genetic manipulations, mutagenesis, multiple tissue culture passage, or multiple passage in a non-native animal host or in eggs.

A "virus" is an encapsidated and/or enveloped virus genome capable of binding to an animal cell and delivering the virus genome to the cell, either to the cytoplasm or to the nucleus, depending on the virus. The term "virus" encompasses both recombinant and non-recombinant viruses. The term "virus" also encompasses both wild type and mutant viruses. Preferred viruses are those originally derived from mammalian cells, and even more preferred viruses are those of human origin.

A "recombinant virus" is a virus which contains one or more genes that are foreign to the wild type virus. Recombinant viruses include, without limitation, those that include a foreign gene such as the human VEGF gene, as well as viruses that comprise other viral genomes.

An "viral vector" is a recombinant virus comprising one or more foreign genes, wherein the viral vector is capable of binding to an animal cell and delivering the foreign gene to the cell.

An "adenovirus genome" is the nucleic acid molecule backbone of an adenovirus particle. The adenovirus genome may contain point mutations, deletions or insertions of nucleotides. The adenovirus genome may further comprise a foreign gene. A "native" or "naturally-occurring" adenovirus genome is one which is isolated from a wild type adenovirus growing in a natural animal host. Although a "naturally occurring" adenovirus genome may be kept and propagated in a laboratory, it has not been intentionally manipulated by, *e.g.*, genetic manipulations, mutagenesis, multiple tissue culture passage, or multiple passage in a non-native animal host or in eggs.

An "adenovirus" is an encapsidated adenovirus genome capable of binding to an animal cell and delivering the adenovirus genome to the cell's nucleus. The term "adenovirus" encompasses both recombinant and non-recombinant adenoviruses. The term "adenovirus" also encompasses both wild

type and mutant adenoviruses. Preferred adenoviruses are those originally derived from mammalian cells, and even more preferred adenoviruses are those of human origin.

A "recombinant adenovirus" is an adenovirus which contains one or more genes that are foreign to a wild type adenovirus. Recombinant adenoviruses include, without limitation, those that include a foreign gene such as the human VEGF gene, as well as adenoviruses that comprise other viral genomes such as the adeno-associated viral (AAV) genome or portions thereof, *e.g.*, hybrid Ad/AAV viruses described elsewhere herein.

An "adenovirus vector" is a recombinant adenovirus comprising one or more foreign genes, wherein the adenovirus vector is capable of binding to an animal cell and delivering the foreign gene to the cell's nucleus.

A "locus" is a site within a virus genome wherein a particular gene normally resides. For instance, the "adenovirus E1 locus" is the site at which the E1 genes reside in the adenovirus genome. If a foreign gene or nucleic acid molecule is inserted into a locus, it may either replace the gene that naturally resides there or it may be inserted at the site within or next to the gene that naturally resides there.

An "adenovirus complementation element" is a nucleic acid molecule which when introduced into a suitable cell by transformation or transfection (producing a complementation cell) is capable of supporting the replication of otherwise replication-incompetent adenovirus. It is important to note that the protein encoded by the complementation element can be a portion of a complete protein (protein fragment) so long as the complementing activity of the complete protein is substantially supplied by the protein fragment.

By "stringent hybridization conditions" is intended 16 hour incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing in 0.1X SSC at about 65°C. By "conditions of low

stringency" is intended 16 hour incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing in 1X SSC, preferably in 3X SSC, even more preferably in 5X SSC at about 65°C, preferably at about 42°C, even more preferably at about 37°C.

By a polynucleotide less than, for example, 90% "identical" to a reference polynucleotide (*e.g.* a wild type, native, or naturally-occurring adenovirus polynucleotide) is intended that the nucleotide sequence is identical to the reference sequence except that the polynucleotide sequence must include an average of ten or more point mutations per each 100 nucleotides of the reference nucleotide sequence. As a practical matter, whether any particular nucleic acid molecule is less than, *e.g.*, 95%, 90%, 80% or 70% identical to, a reference nucleotide sequence shown can be determined conventionally using known computer programs such as the Bestfit program using default parameters (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2: 482-489, 1981) to find the best segment of homology between two sequences. Once Bestfit determines the most optimal alignment between two nucleotide sequences it is then a simple determination to count the number of base changes necessary to transform the nucleotide sequence into the reference nucleotide sequence.

"RCA-free" according to the present invention means less than about 1 pfu of replication competent adenovirus (RCA) in approximately 10^9 preferably 10^{10}, 10^{11}, 10^{12}, or 10^{13} pfu of an rAd preparation.

The term "a" or "an" entity, refers to one or more of that entity; for example, "a nucleic acid molecule," is understood to represent one or more nucleic acid molecules. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

Construction of Complementing Cell Lines

Target Cells

Target cells, or host cells, which may be recombinantly engineered to complement replication-incompetent recombinant adenovirus, or other replication-incompetent virus, may be selected from any mammalian species including, without limitation, human diploid cells such as MRC-5, WI-38, HER, HEL, HEK, A549 and human aneuploid cells such as HeLa. Cells isolated from other mammalian species are also useful, for example, primate cells, rodent cells or other cells commonly used in biological laboratories. Among such primate cell types are diploid Vero, CV-1 and FRhL cells. The selection of the mammalian species providing the cells, as well as their euploid type, is not a limitation of this invention; nor is the type of mammalian cell, *i.e.*, fibroblast, hepatocyte, tumor cell, etc.

In one preferable embodiment the WI-38 or MRC-5 cell is used as the target cell. These lines, and WI-38 in particular, have been used for many years in vaccine production. They have a long safety record suggesting that no harmful adventitious agents are resident in these cell lines. Thus, the WI-38 or MRC-5 cell is a preferable choice for production of recombinant adenovirus to be used in a clinical setting.

Adenovirus Nucleic Acid Molecules and Complementation Elements

Suitably, the packaging cells are stably transformed or transfected with a complementation element comprising a nucleic acid molecule carrying, at a minimum, nucleotide sequences encoding one or more functional adenovirus proteins which are absent from or are not functionally encoded by the replication-defective adenovirus vector of choice. It is known in the art that the adenovirus loci E1, E2a and E4ORF6 are essential for viral replication. Accordingly, the complementation element contains nucleotide sequences encoding one or more adenovirus proteins, preferably one or more essential adenovirus proteins, even

more preferably one or more proteins encoded by the E1 locus and most preferably all of the proteins encoded by the Ad5 E1 locus including the Ad5 8.3 kDa E1b protein.

As a means of avoiding homology with the recombinant Ad vector and concomitantly reducing the likelihood of homologous recombination leading to RCA production, the complementation element of the present invention contains a non-naturally occurring adenovirus nucleotide sequence.

Thus, in one embodiment, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, wherein the sequence of said polynucleotide is not a naturally-occurring adenovirus nucleotide sequence. What is meant by "not a naturally-occurring adenovirus sequence" is a nucleotide sequence which is not a native adenovirus sequence, *i.e.*, it is not the exact nucleotide sequence which encodes the at least five amino acids in a native adenovirus genome, but because of the degeneracy of the genetic code, it encodes the same 5 amino acids. Since the genetic code is well known it would be routine for one of skill in the art to contemplate any number of nucleotide sequences (literally thousands upon thousands) which are non-naturally occurring but which code for a known adenovirus amino acid sequence. Such a list could be computer generated but will not be presented herein in the interest of economy.

According to this embodiment, an isolated nucleic acid molecule is provided comprising a polynucleotide which encodes at least five amino acids of a naturally-occurring polypeptide from any adenovirus, including, but not limited to, a polypeptide from a human adenovirus, an avian adenovirus, a bovine adenovirus, a feline adenovirus, a canine adenovirus, a murine or other rodent adenovirus, a fish adenovirus, a porcine adenovirus, a caprine adenovirus, an ovine adenovirus, an equine adenovirus, or a simian adenovirus, but where the polynucleotide sequence is not a naturally-occurring adenovirus nucleotide sequence. Nucleotide and amino acid sequences derived from each of these categories of adenoviruses can be obtained through GenBank.

In a preferred embodiment of the invention, an isolated nucleic acid molecule is provided comprising a polynucleotide which encodes at least five amino acids of a naturally-occurring polypeptide from one of the known human adenovirus types 1-46, but where the polynucleotide sequence is not a naturally-occurring human adenovirus nucleotide sequence. All human adenovirus types 1-46 are available from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassass, VA 20110-2209, and the nucleotide and amino acid sequences for can be obtained through GenBank.

In a more preferred embodiment of the invention, a nucleic acid molecule is provided comprising a polynucleotide which encodes at least 5 amino acids of a naturally occurring Ad5 or human adenovirus type 2 (Ad2) polypeptide, but where the polynucleotide sequence is not a naturally-occurring human Ad5 or Ad2 nucleotide sequence. The complete nucleotide sequences of Ad2 and Ad5 are available from GenBank, *see e.g.*, GenBank accession no. M73260 for the human adenovirus type 5 sequence, and J01917 for the human adenovirus type 2 sequence.

A non-naturally occurring polynucleotide sequence as referred to above may encode as few as 5 contiguous amino acids of a naturally occurring adenovirus polypeptide. A non-naturally occurring polynucleotide sequence preferably encodes at least 6 contiguous amino acids of a naturally occurring adenovirus polypeptide, more preferably at least 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 400, or 500 contiguous amino acids of a naturally occurring adenovirus polypeptide and most preferably the polynucleotide sequence encodes an entire adenovirus polypeptide.

A naturally occurring adenovirus polypeptide as referred to above is any adenovirus polypeptide, preferably an essential adenovirus polypeptide, more preferably an E1, E2 or E4 polypeptide, even more preferably an E1 polypeptide, and most preferably an Ad5 E1b polypeptide selected from the group consisting of the 55 kDa E1b polypeptide, the 21 kDa E1b polypeptide, and the 8.3 kDa E1b polypeptide.

A non-naturally occurring polynucleotide sequence as referred to above may contain as few as three (3) nucleotide substitutions, preferably it contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 60, 75, 100, 125, 150, 175, 200, 250, 300, or more nucleotide substitutions, each as compared to a corresponding native adenovirus nucleotide sequence, preferably as compared to a native human adenovirus sequence, and even more preferably as compared to a native Ad5 nucleotide sequence.

In another embodiment, an isolated nucleic acid molecule is provided comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, where the polynucleotide will not hybridize to a naturally occurring adenovirus genome under stringent conditions. Preferably, such a polynucleotide will not hybridize to a naturally occurring human adenovirus genome under stringent conditions. More preferably, such a polynucleotide will not hybridize to a naturally occurring Ad5 genome under stringent conditions. A preferred embodiment provides an isolated nucleic acid molecule comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, where the polynucleotide will not hybridize to a naturally occurring adenovirus genome under conditions of low stringency. Preferably, such a polynucleotide will not hybridize to a naturally occurring human adenovirus genome under conditions of low stringency. Most preferably, such a polynucleotide will not hybridize to a naturally occurring Ad5 genome under conditions of low stringency.

Two general approaches have been used to study the sequence similarlity requirements for DNA recombination in mammalian cells. One is to measure the rate of recombination as a function of the length of identical nucleotides shared between two polynucleotides. As one of ordinary skill in the art would readily appreciate, sequence "identity" in this context refers to double stranded polynucleotides, and contemplates interactions between the complementary single stranded components of one or more double stranded polynucleotides. Using this approach, it has been shown that in mammalian cells the rate of intra-

chromosomal recombination between two closely matched sequences drops off sharply when the length of complete sequence identity is reduced from 295 to 200 bp (Liskay, R., *et al.*, *Genetics 115*:161-167 (1987)). Alternatively, the effect of nucleotide mismatches on the rate of recombination can be measured. Using this strategy, it has been determined that intra-chromosomal recombination in mammalian cells between two linked sequences which are 81% identical was reduced over 1000-fold relative to recombination between sequences displaying near perfect identity (Waldman, A. *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 84*:5340-5344 (1987)) and that synergistically large decreases in recombination can result from relatively small changes in degree of sequence identity (Lukacsovich, T. *et al.*, *Genetics*, *151*:1559-1568 (1999)). It has been shown that efficient extra-chromosomal recombination in mammalian cells requires a length of identical nucleotides of about 200 bp (Rubnitz, J. *et al.*, *Mol. Cell. Biol. 4*:2253-2258 (1984)).

While not being bound by theory, it is believed by the inventors that as few as 3 base pair substitutions evenly dispersed in 100 nucleotides of coding sequence will significantly reduce homologous recombination. Accordingly, another embodiment of the invention provides an isolated nucleic acid molecule comprising a polynucleotide, where the sequence of the polynucleotide is at least about 60% identical to, but is less than about 97% identical to a naturally occurring adenovirus polynucleotide sequence of the same length. Preferably the polynucleotide sequence is at least about 60% identical to, but is less than about 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75%, 70% or 65% identical to a naturally occurring adenovirus polynucleotide sequence of the same length, preferably a human adenovirus, more preferably Ad5. Preferably, the polynucleotide sequence identity is reduced through the introduction of silent mutations of nucleotides at degenerate positions within codons such that the translational coding capacity of the nucleotide sequence is not altered. More preferably the polynucleotide encodes at least 5, preferably at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 75, 100, 125, 150, 175,

200, 250, or 300, contiguous amino acids of a naturally-occurring adenovirus polypeptide.

Optimally, such silent mutations are evenly dispersed across the full length of the polynucleotide. Accordingly, in one embodiment a silent mutation is present at least once in every ten contiguous codons, preferably at least one silent mutation is present in every 9, 8, 7, 6, 5, 4 or 3 contiguous codons. In a related embodiment, the polynucleotide sequence contains fewer than 200 contiguous nucleotides of wild-type adenovirus sequence, preferably fewer than 150, 125, 100, 75, 60, 50, 40, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7 or 6 contiguous nucleotides of wild-type adenovirus sequence. Most preferably the nucleotide sequence contains as little sequence identity with the corresponding wild-type adenovirus sequence as possible (*i.e.* most fully degenerate) yet retains the translational coding capacity of the wild-type adenovirus sequence.

In certain embodiments, it is preferred that the polynucleotide encode polypeptides capable of complementing an RCA defective in the Ad E1 proteins. A preferred example of this embodiment provides an isolated nucleic acid molecule comprising a polynucleotide, where the sequence of the polynucleotide is at least about 60% identical to, but is less than about 97% identical to a naturally occurring Ad5 polynucleotide comprising nucleotides 1 to 198 of SEQ ID NO:20, or nucleotides 287 to 301 of SEQ ID NO:20. Preferably the polynucleotide sequence is at least about 60% identical to, but is less than about 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75%, 70% or 65% identical to a naturally occurring Ad5 polynucleotide comprising nucleotides 1 to 198 of SEQ ID NO:20, or nucleotides 287 to 301 of SEQ ID NO:20. Preferably, the polynucleotide sequence identity is reduced through the introduction of silent mutations of nucleotides at degenerate positions within codons such that the translational coding capacity of the nucleotide sequence is not altered. More preferably the polynucleotide encodes at least 5, preferably at least 6, 7, 8, 9, 10,

11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 75, 100, 125, 150, 175, 200, 250, or 300, contiguous amino acids of an Ad5 E1 polypeptide.

Optimally, such silent mutations are evenly dispersed across the full length of the polynucleotide. Accordingly, in one embodiment a silent mutation is present at least once in every ten contiguous codons of the polynucleotide comprising nucleotides 1 to 198 of SEQ ID NO:20, preferably at least one silent mutation is present in every 9, 8, 7, 6, 5, 4 or 3 contiguous codons of the polynucleotide comprising nucleotides 1 to 198 of SEQ ID NO:20. In another embodiment a silent mutation is present at least once in 1, 2, 3, 4, or 5 of the contiguous codons of the polynucleotide comprising nucleotides 287 to 301 of SEQ ID NO:20. In a related embodiment, the polynucleotide sequence contains fewer than 200 contiguous nucleotides of wild-type adenovirus sequence, preferably fewer than 150, 125, 100, 75, 60, 50, 40, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7 or 6 contiguous nucleotides of wild-type adenovirus sequence. Most preferably the nucleotide sequence contains as little sequence identity with the corresponding wild-type adenovirus sequence as possible (*i.e.* most fully degenerate) yet retains the translational coding capacity of the wild-type adenovirus sequence.

In another desirable embodiment, the invention provides a nucleic acid molecule comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, wherein the codons encoding the naturally-occurring adenovirus polypeptide are known to be preferential for translation in a given eukaryotic cell, preferably a mammalian cell, more preferably preferential for translation in a human cell, and even more preferably preferential for translation in a human WI-38 or MRC-5 cell.

Methods to choose codons which will preferentially be translated in a given species are well known to those of skill in the art. For example, to choose codons which are preferentially utilized in human cells, a codon usage table for sequenced human DNA is used. A suitable table for human codon usage is shown as Table 1. The amino acid to be encoded is found in the first column, and

the codon for that amino acid which has the highest fraction of usage in the species is chosen. For example, the skilled artisan, in order to optimize the expression of a nucleotide sequence encoding the amino acid valine in humans would utilize the codon GTG, which is used 48% of the time in human genes.

Table 1

***Homo sapiens* [gbpri]: 17625 CDS's (8707603 codons)**

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	144066.00	16.54	0.25
Gly	GGA	143152.00	16.44	0.25
Gly	GGT	94776.00	10.88	0.16
Gly	GGC	201492.00	23.14	0.35
Glu	GAG	353389.00	40.58	0.59
Glu	GAA	248945.00	28.59	0.41
Asp	GAT	193421.00	22.21	0.46
Asp	GAC	230449.00	26.47	0.54
Val	GTG	255326.00	29.32	0.48
Val	GTA	59454.00	6.83	0.11
Val	GTT	93237.00	10.71	0.17
Val	GTC	128872.00	14.80	0.24
Ala	GCG	66830.00	7.67	0.11
Ala	GCA	135692.00	15.58	0.22
Ala	GCT	160540.00	18.44	0.26
Ala	GCC	249413.00	28.64	0.41
Arg	AGG	96943.00	11.13	0.20
Arg	AGA	97769.00	11.23	0.20
Ser	AGT	101784.00	11.69	0.15
Ser	AGC	168393.00	19.34	0.24
Lys	AAG	288839.00	33.17	0.58
Lys	AAA	205438.00	23.59	0.42
Asn	AAT	146576.00	16.83	0.45
Asn	AAC	176150.00	20.23	0.55
Met	ATG	193713.00	22.25	1.00
Ile	ATA	60712.00	6.97	0.15
Ile	ATT	137003.00	15.73	0.35
Ile	ATC	194225.00	22.31	0.50
Thr	ACG	56070.00	6.44	0.12
Thr	ACA	128130.00	14.71	0.27
Thr	ACT	110775.00	12.72	0.24
Thr	ACC	173197.00	19.89	0.37
Trp	TGG	112968.00	12.97	1.00
End	TGA	11388.00	1.31	0.51
Cys	TGT	84611.00	9.72	0.44
Cys	TGC	107993.00	12.40	0.56
End	TAG	4674.00	0.54	0.21
End	TAA	6412.00	0.74	0.29

Tyr	TAT	105741.00	12.14	0.43
Tyr	TAC	142002.00	16.31	0.57
Leu	TTG	104842.00	12.04	0.12
Leu	TTA	60542.00	6.95	0.07
Phe	TTT	144711.00	16.62	0.44
Phe	TTC	180549.00	20.73	0.56
Ser	TCG	39565.00	4.54	0.06
Ser	TCA	99242.00	11.40	0.14
Ser	TCT	126354.00	14.51	0.18
Ser	TCC	153781.00	17.66	0.22
Arg	CGG	100931.00	11.59	0.21
Arg	CGA	53982.00	6.20	0.11
Arg	CGT	40995.00	4.71	0.08
Arg	CGC	95930.00	11.02	0.20
Gln	CAG	299873.00	34.44	0.75
Gln	CAA	102442.00	11.76	0.25
His	CAT	88133.00	10.12	0.41
His	CAC	129455.00	14.87	0.59
Leu	CTG	348202.00	39.99	0.41
Leu	CTA	58998.00	6.78	0.07
Leu	CTT	108127.00	12.42	0.13
Leu	CTC	168315.00	19.33	0.20
Pro	CCG	61627.00	7.08	0.12
Pro	CCA	143936.00	16.53	0.27
Pro	CCT	149994.00	17.23	0.28
Pro	CCC	176517.00	20.27	0.33

Coding GC 52.75% 1st letter GC 56.12% 2nd letter GC 42.36% 3rd letter GC 59.77%
Genetic code 1: Standard

Similar tables are available for other species and are available, *e.g.*, on the internet at < http://www.kazusa.or.jp/codon/ > (visited April 10, 2000).

In a particularly preferred embodiment, the invention comprises a nucleic acid molecule comprising at least 20 contiguous nucleotides of the sequence shown as nucleotides 1 to 198 of SEQ ID NO:1, preferably at least 30, 40, 50 or 100 contiguous nucleotides of the sequence shown as nucleotides 1 to 198 SEQ ID NO:1, more preferably the entire sequence shown as SEQ ID NO:1. SEQ ID NO:1 corresponds to a region of the E1 locus of Ad5 extending from about nucleotide 3309 to about nucleotide 3614 of the complete Ad5 genome (GenBank Accession No. M73260, fragment depicted herein as SEQ ID NO:20), and encodes for C-terminus of the E1b 55 kD protein and the 3' exon of the 8.3 kD E1b protein. Construction of the complementation element is described in Example 1(A) and Example 7. The sequence was modified to contain all possible

silent mutations in the coding regions, as shown in Figure 2. Where possible, the base substitutions were chosen to correspond to the most frequently used codons in the human codon usage table shown in Table 1.

In another preferred embodiment, the invention comprises a nucleic acid molecule comprising at least 20 contiguous nucleotides of the sequence shown as nucleotides 1 to 198 of SEQ ID NO:18, preferably at least 30, 40, 50 or 100 contiguous nucleotides of the sequence shown as nucleotides 1 to 198 SEQ ID NO:18, more preferably the entire sequence shown as SEQ ID NO:18. SEQ ID NO:18 also corresponds to a region of the E1 locus of Ad5 extending from about nucleotide 3309 to about nucleotide 3614 of the complete Ad5 genome (GenBank Accession No. M73260, fragment depicted herein as SEQ ID NO:20). Construction of the complementation element is described in Example 1(C) and Example 7. The sequence was modified to contain silent mutations evenly spaced at about 15 nucleotides apart, as shown by the underlined nucleotide substitutions in in Figure 2. Where possible, the base substitutions were chosen to correspond to the most frequently used codons in the human codon usage table shown in Table 1.

In yet another embodiment of the invention, a nucleic acid molecule is provided which comprises a sequence selected from the group consisting of: (a) nucleotides 1 to 198 of SEQ ID NO:1; (b) nucleotides 298 to 312 of SEQ ID NO:1; and (c) nucleotides 1 to 315 of SEQ ID NO:1.

In yet embodiment of the invention, a nucleic acid molecule is provided which comprises a sequence selected from the group consisting of: (a) nucleotides 1 to 198 of SEQ ID NO:18; (b) nucleotides 298 to 312 of SEQ ID NO:18; and (c) nucleotides 1 to 315 of SEQ ID NO:18.

In yet another embodiment of the invention, a nucleic acid molecule is provided which comprises a sequence selected from the group consisting of: (a) nucleotides 298 to 312 of SEQ ID NO:19; and (b) nucleotides 1 to 315 of SEQ ID NO:19. SEQ ID NO:19 also corresponds to a region of the E1 locus of Ad5 extending from about nucleotide 3309 to about nucleotide 3614 of the complete

Ad5 genome (GenBank Accession No. M73260, fragment depicted herein as SEQ ID NO:20). Construction of the complementation element is described in Example 1(B) and Example 7. The sequence was has the wild-type Ad5 sequence in the 55 kD coding region, but has all the possible base substitutions in the 3' exon of the 8.3 kD coding region, as shown by the boxed nucleotide substitutions in Figure 2. Where possible, the base substitutions were chosen to correspond to the most frequently used codons in the human codon usage table shown in Table 1.

In another embodiment, the invention provides a complementation element which encodes an essential adenovirus protein wherein the complementation element comprises a nucleic acid molecule described herein. Preferably the complementation element comprises an Ad E1 locus, more preferably a human Ad E1 locus, even more preferably an Ad5 E1 locus.

Another way to reduce or eliminate homologous recombination leading to RCA is to substitute one or more heterologous introns for naturally-occurring adenovirus introns. The genome structure of the adenovirus, including the location of naturally occurring introns, is well known (see, The Adenoviruses, Ed. Harold Ginsberg, 1984, Plenum Press, NY, for a review). The particular heterologous intron used in this embodiment of the invention is not deemed critical to the invention and is therefore not limiting, however, several examples of heterologous introns are provided below for the convenience of the reader. Introns for use in the invention include the SV40 late region intron, the SV40 T intron, the human cytomegalovirus immediate early intron-A, immunoglobulin introns, and many others well known to those of skill in the art. In addition, a heterologous intron can be a hybrid of one or more heterologous and/or native adenovirus introns. For example, a hybrid intron which contains 5' and 3' splice sites, as well as central branch sites, from different intervening sequences, or which are engineered to be consensus splice donor and splice acceptor sites. Such consensus splice sites are disclosed in P.C.T. Publication No. WO 99/29848, which is incorporated herein by reference in its entirety. Thus, the nucleic acid

molecule of the invention and the complementation element of the invention may further comprise a heterologous intron and/or splice sites with or without a non-naturally occurring coding sequence as described above.

As described above, a complementation element which encodes all of the E1 proteins is a preferred embodiment of the present invention. Figure 1 shows a diagrammatic representation of a portion of a representative E1 complementation element constructed according to the method of Example 1. Preferably, E1 complementation elements according to the invention lack adenovirus sequences 5' of the E1 region, preferably excluding the native E1a promoter since some first generation adenovirus vectors contain part or all of the E1a promoter. The E1 complementation element of the invention preferably does not comprise more than 14 contiguous nucleotides of wild-type Ad nucleotide sequence corresponding to the region from about nt 3,000 to 4,300 of the Ad5 genome, preferably from about nt 3,300 to about 3,700 of the Ad5 genome and most preferably between about nt 3,400 and 3,500.

As will be appreciated by those of skill in the art, the adenovirus vector also may advantageously be modified by alternative codon usage and/or through the replacement of wild-type introns for heterologous introns. As stated elsewhere herein, alteration of vector sequences is less preferable than alteration of sequences in production materials (such as the packaging line) since the vector itself forms part of the therapeutic drug and any modification of the drug itself would require extensive preclinical and clinical testing for safety and efficacy. Modification of the adenovirus vector is nonetheless useful, for example, an adenovirus vector may be used as a helper virus in the production of AAV. See, for example, US Patent No. 5,871,982, incorporated herein by reference in its entirety.

Regulatory Elements and Vectors

The complementation element may be in any form which effects the stable transfer of the nucleic acid molecule to the target cell and expression of the encoded protein by the target cell. Most suitably, the complementation element is carried in a vector. It may be stably integrated into the host cell chromosome or exist as an episomal, extra-chromosomal element (see, *e.g.*, Caravokyri, *et al.*, *J. Virol., 69*:6627-6633 (1995)). A "vector" includes, without limitation, any construct which can carry and transfer genetic information, such as a plasmid, a phage, a transposon, a cosmid, a chromosome, a virus, a virion, a replicon, and a messenger RNA. Preferred vectors include chromosomal, episomal and virus-derived vectors *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, and from yeast chromosomal elements; from insect viruses such as baculoviruses, from papova viruses such as SV40, from pox viruses such as vaccinia viruses and fowl pox viruses, from adenoviruses, from herpesviruses such as herpes simplex virus, bovine herpesvirus and pseudorabies virus, and from retroviruses; and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. Generally, any vector suitable to maintain, propagate or express polynucleotides or to express a polypeptide in a host may be used for expression in this regard. In one particularly suitable embodiment, the complementation element is placed in a plasmid vector.

The present invention also relates to vectors which include any of the isolated nucleic acid molecules of the present invention, *e.g.*, a nucleic acid molecule comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, wherein the sequence of said polynucleotide is not a naturally-occurring adenovirus nucleotide sequence; and to host cells comprising the vectors. Such vectors and host cells are useful for, *e.g.*, amplification of a complementation element, construction of a

complementation element, expression of complementation polypeptides, and propagation of replication defective viruses.

Vectors may be introduced into host cells using well known techniques such as infection, transduction, transfection, transvection, lipofection, electroporation, microinjection, particle bombardment, and transformation. Generally, a plasmid vector is introduced into a cell by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, or electroporation. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods in Molecular Biology (1986).

Nucleic acid molecules of the present invention may be joined to a vector containing a selectable marker for propagation in a host. Such markers include dihydrofolate reductase, tetracycline resistance, hygromycin resistance, or neomycin resistance for eukaryotic cell culture and tetracycline, chloramphenicol, or ampicillin resistance genes for culturing in *E. coli* and other bacteria. In certain preferred embodiments in this regard, the vectors provide for specific expression, which may be inducible and/or cell type-specific. As indicated, vectors will preferably include at least one selectable marker. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

The nucleic acid molecule should be operably associated with an appropriate promoter, such as prokaryotic promoters, *e.g.*, the phage lambda PL and PR promoters as well as other bacteriophage promoters such as T3, T7, T7/*lac*, SP6, SP01, the *E. coli lac*I, *lac*Z, *gpt*, *trp*, *trc*, *oxy-pro*, *omp/lpp*, *rrn*B, and *tac* promoters, and eukaryotic promoters, *e.g.*, metallothionein, such as the mouse metallothionein-I promoter, alpha-mating factor, actin, heat shock, tissue specific promoters such as lymphokine-inducible promoters, *Pichia* alcohol oxidase, alphvirus subgenomic promoter, human cytomegalovirus immediate early promoter, with or without intron A, the HSV thymidine kinase promoter,

the SV40 early and late promoters, and promoters of retroviral LTRs such as from Rous Sarcoma virus. the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV). Other suitable promoters will be known to the skilled artisan.

Transcription of a nucleic acid molecule of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

In general, expression constructs will contain sites for transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

Representative examples of appropriate hosts for amplification of the nucleic acid molecules of the invention include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as *Saccharomyces* and *Pichia* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, BHK, and Bowes melanoma cells; and plant cells. Appropriate culture media and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1

and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Once the desired nucleic acid molecule (complementation element) is engineered, it may be transferred to the target host cell by any suitable method. Such methods include, for example, transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. Thereafter, cells are cultured according to standard methods and, optionally, seeded in media containing an antibiotic to select for cells containing the cells expressing the resistance gene. After a period of selection, the resistant colonies are isolated, expanded, and screened for expression of the encoded adenovirus protein. See, Sambrook *et al*, *supra*. Alternatively, the newly created complementing cells are infected with recombinant E1-deleted adenovirus carrying a reporter gene, such as the *E. coli* β-galactosidase gene, and selected on the basis of their ability to complement replication-incompetent adenovirus replication.

Systems for the Production of RCA-free Adenovirus

The invention provides combinations of adenovirus vectors and complementing producer cell lines, collectively "systems". Most broadly, the invention encompasses a system for the production of adenovirus particles comprising: (a) a recombinant replication-incompetent adenovirus vector; and (b) a complementing cell which encodes those essential gene products which are not expressed by the replication-incompetent adenovirus vector, but which is incapable of homologous recombination with the replication-incompetent adenovirus vector.

Preferably, the invention provides producer complementing cell lines which may be combined with those replication-incompetent adenovirus vectors with genomes containing partial fragments of essential genes, even though those genes are not expressed. These adenovirus vectors have posed special problems,

because the standard complementing cell lines, in order to provide the needed essential gene product, must necessarily contain significant overlapping coding regions with the vector. Accordingly, preferred complementing cell lines of the present invention comprise a polynucleotide at least 15 nucleotides in length encoding at least 5 contiguous amino acids of a naturally-occurring adenovirus polypeptide, where a coding region encoding the same amino acids is contained on the replication-incompetent adenovirus vector. However, due to the degeneracy of the genetic code, the sequence of the polynucleotide in the complementing cell line is not the naturally-occurring adenovirus nucleotide sequence found in the adenovirus genome. Preferably the complementing cell line comprises a polynucleotide at least 20, 25, 30, 40, 50, 60, 75, 100, 150, 200, 250, 500, or 1,000 nucleotides in length, encoding at least 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, or 330 amino acids of a naturally occurring adenovirus polypeptide, where a coding region encoding one or more of the same amino acids is contained on the replication-incompetent adenovirus vector, but, due to the degeneracy of the genetic code, the sequence of the polynucleotide in the complementing cell line is not the naturally-occurring adenovirus nucleotide sequence found in the adenovirus genome.

Preferably the sequence of the polynucleotide in the complementing cell line and the sequence of the replication-incompetent adenovirus genome, although encoding at least 5 identical consecutive amino acids, are less than 97%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% identical to one another. Also, preferably the sequence of the polynucleotide in the complementing cell line and the sequence of the replication-incompetent adenovirus genome which encode at least 5 identical consecutive amino acids are incapable of hybridizing to one another at stringent conditions. More preferably the two sequences will not hybridize at conditions of low stringency.

It is important to point out here that systems of the invention are not limited to cells or viral vectors comprising non-naturally occurring nucleotide sequences which encode wild-type adenovirus polypeptide sequences. There is

significant nucleotide sequence variation between different strains of adenovirus. Thus, matched complementation elements and vectors may contain naturally occurring adenovirus sequences which would be identical but for the degeneracy of the genetic code.

In one preferred embodiment the vector has only naturally occurring adenovirus sequences and homology with the complementation element/complementing cell line is reduced by the introduction into the complementation element/complementing cell line of a polynucleotide with non-adenovirus nucleic acid sequence, but which nonetheless provides the coding region for a naturally-occurring adenovirus polypeptide.

In another embodiment, the complementation element/complementing cell line has only naturally occurring adenovirus sequences and homology is reduced by the introduction into the vector of a polynucleotide with non-adenovirus nucleic acid sequence, but which nonetheless provides the coding region for a naturally-occurring adenovirus polypeptide. Alternatively in this embodiment, the polynucleotide with the non-adenovirus nucleic acid sequence need not provide coding region for a naturally occurring adenovirus polypeptide, if that polypeptide is provided in trans by the complementing cell line.

The polynucleotide with a non-adenovirus nucleotide sequence contained in either the vector or the cell line of a system herein may be any non-adenovirus sequence described elsewhere herein. Likewise, the system may comprise any combination of cell and vector described elsewhere herein which: (a) can be used together to produce replication-incompetent adenovirus particles; and (b) would have overlapping sequence identity but for the degeneracy of the genetic code.

In any of the systems described herein, the complementing cell line or the replication incompetent vector may also comprise a heterologous intron in place of a naturally-occurring adenovirus intron, where the heterologous intron may further comprise heterologous consensus splice donor and splice acceptor regions.

In a preferred embodiment, the system of the invention comprises: (a) a complementing cell comprising a polynucleotide encoding the 55 kDa E1b

protein of Ad5, at least 50 contiguous nucleotides of which is non-adenovirus sequence; and (b) an adenovirus vector containing at least 50 contiguous nucleotides of wild-type Ad5 sequence between nucleotides 3311 and 3614.

Also preferred is a system comprising (a) a complementing cell line comprising a polynucleotide having the sequence of SEQ ID NO:1, SEQ ID NO:18, or SEQ ID NO:19; and (b) an adenovirus vector containing at least 50 contiguous nucleotides of wild-type Ad5 sequence between nucleotides 3311 and 3614.

Use of the Complementing Cells

The complementing cells of the invention are useful for a variety of purposes. Most suitably, the cells are used in high yield production of recombinant replication-incompetent adenovirus vectors (*i.e.* adenovirus particles) in the absence of detectable RCA.

Replication and Packaging of Replication Incompetent Ad Vectors

In a preferred embodiment, the present invention provides a method of packaging of an Ad vector deleted of E1, E2, and/or E4 (collectively "replication-deficient rAd" or "replication-incompetent rAd"), containing a transgene, into an adenovirus particle useful for delivery of the transgene to a host cell. In a preferred embodiment, the replication deficient rAd vector contains all adenovirus genes necessary to produce and package an infectious adenovirus particle when replicated in the presence of complementing proteins, *e.g.*, such as are supplied by the cell lines of the invention. Preferred vectors for use in this aspect of the invention are: (a) the first-generation Ad vector which contains defects in both the E1a and E1b sequences, and most desirably, is deleted of all or most of the sequences encoding these proteins; (b) the second-generation Ad vector which contains defects in the E1a, E1b and E2a sequences; (c) the third-

generation Ad vector which contains defects in the E1 and E4 sequence, and most desirably is deleted of all or most of the sequence encoding these proteins; and (d) the fourth-generation Ad vector which is completely devoid of all viral genes.

At a minimum, the E1-deleted vector to be packaged contains adenovirus 5' and 3' cis-elements necessary for replication and packaging, a transgene, and a pIX gene or a functional fragment thereof. The vector further contains regulatory sequences which permit expression of the encoded transgene product in a host cell, which regulatory sequences are operably associated with the transgene. Also included in the vector are regulatory sequences operably associated with other gene products, *e.g.*, the pIX gene, carried by the vector.

Thus, a method of producing recombinant replication-incompetent adenovirus particles is provided comprising the steps of: (a) infecting or transfecting a complementing cell line of the invention with a replication incompetent adenovirus vector; and (b) purifying the adenovirus particles. Compositions comprising substantially pure preparations of adenovirus particles produced by the above-described method are also provided. It is not necessary that the complementation element/complementing cell line and the replication incompetent adenovirus vector contain any related sequences whatsoever, however, and advantage of the present invention is that existing adenovirus vectors, which do contain related sequences, *e.g.* in the E1 locus, are suitable for use in the present invention. Accordingly, in certain preferred embodiments, the replication incompetent adenovirus vector comprises at least 15 consecutive nucleotides which, but for the degeneracy of the genetic code, would be identical to at least 15 consecutive nucleotides in the complementation element/complementing cell line.

Adenovirus Elements. The replication incompetent vector to be packaged includes, at a minimum, adenovirus cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain. These are 5' and 3' cis-elements

necessary for packaging linear Ad genomes and further contain the enhancer elements for the E1 promoter.

The E1-deleted vector to be packaged into a viral particle is further engineered so that it expresses the pIX gene product. Most suitably, the pIX gene is intact, containing the native promoter and encoding the full length protein. According to Babiss and Vales, *J. Virol. 65*:598-605 (1991), the native pIX promoter begins at Ad5 nucleotide 3525, which overlaps with the E1 region by about 86 nucleotides (see Figure 2). However, where desired, the native pIX promoter may be substituted by another desired promoter. Alternatively, sequences encoding a functional fragment of pIX may be selected for use in the vector. In yet another alternative embodiment, the native sequences encoding pIX or a functional fragment thereof may be modified to enhance expression. For example, the native sequences may be modified, *e.g.*, by site-directed mutagenesis or another suitable technique, to insert optimized codons to enhance expression in a selected host cell. Suitable codons to utilize for any given host cell may be determined by use of a codon usage table for the species of the cell line, for example a human codon usage table as shown in Table 1, *supra*.

In a suitable embodiment, the adenovirus sequences in the E1-deleted vector include the 5' and 3' cis-elements, functional E2 and E4 regions, intermediate genes IX and IXa, and late genes L1 through L5. However, the E1-deleted vector may be readily engineered by one of skill in the art, taking into consideration the minimum sequences required, and is not limited to these exemplary embodiments.

The vector is constructed such that the transgene and the sequences encoding pIX are located downstream of the 5' ITRs and upstream of the 3' ITRs. The transgene is a heterologous polynucleotide, which encodes a polypeptide, protein, or other product, of interest. The transgene is operably associated with regulatory components in a manner which permits transgene transcription.

Transgene. The composition of the transgene will depend upon the use to which the resulting adenovirus vector will be put. For example, one type of transgene is a reporter gene, which upon expression produces a detectable signal. Such reporter genes include without limitation, polynucleotides encoding β-lactamase, β-galactosidase, alkaline phosphatase, thymidine kinase, green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), and luciferase. Other transgenes of interest include membrane bound proteins including, for example, CD2, CD4, CD8, the influenza hemagglutinin protein, and others well known in the art, to which high affinity antibodies directed thereto exist or can be produced by conventional means, and fusion proteins comprising a membrane bound protein appropriately fused to an antigen tag domain from, among others, hemagglutinin or Myc.

Desirably, however, the transgene is a non-marker sequence encoding a product which is useful in biology and medicine, such as proteins, peptides, antisense nucleic acids (*e.g.* RNAs), enzymes, or catalytic RNAs. The transgene may be used to correct or ameliorate gene deficiencies, which may include deficiencies in which normal genes are expressed at less than normal levels or deficiencies in which the functional gene product is not expressed.

A preferred type of transgene sequence encodes a therapeutic protein or polypeptide which is expressed in a host cell. Suitable therapeutic proteins or polypeptides include, but are not limited to granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), interleukin 2 (IL-2), interleukin-3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 15 (IL-15), interleukin 18 (IL-18), interferon alpha (IFNα), interferon beta (IFNβ), interferon gamma (IFNγ), interferon omega (IFNω), interferon tau (IFNτ), interferon gamma inducing factor I (IGIF), transforming growth factor beta (TGF-β), RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage

inflammatory proteins (*e.g.*, MIP-1 alpha and MIP-1 beta), *Leishmania* elongation initiating factor (LEIF), platelet derived growth factor (PDGF), TNF, growth factors, *e.g.*, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, (FGF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-2 (NT-2), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), neurotrophin-5 (NT- 5), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), erythropoietin (EPO), and insulin.

The invention further includes using multiple transgenes, *e.g.*, to correct or ameliorate a gene defect caused by a combination or proteins or by a multisubunit protein. In certain situations, a different transgene may be used to encode each subunit of a protein, or to encode different peptides or proteins. This is desirable when the size of the DNA encoding the protein subunit is large, *e.g.*, for an immunoglobulin, the platelet-derived growth factor (PDGF), or a dystrophin protein. In order for a cell to produce the multisubunit protein, the cell is infected with separate recombinant viruses containing each of the different subunits. Alternatively, different subunits of a protein may be encoded by the same transgene. In this case, a single transgene includes a polynucleotide encoding all of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). This is desirable when the size of the polynucleotide encoding all of the subunits is small, *e.g.*, the total length of the polynucleotide encoding all of the subunits and the IRES is less than about five kb. Other useful gene products include, non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions. For example, single-chain engineered immunoglobulins could be useful in certain immunocompromised patients. Other types of non-naturally occurring gene sequences include antisense molecules and catalytic nucleic acids, such as ribozymes, which could be used to reduce overexpression of a gene. However,

the selected transgene may encode any product desirable for study. The selection of the transgene sequence is not a limitation of this invention.

Expression Control Sequences. In addition to the major elements identified above for the adenovirus vector (*e.g.* the adenovirus sequences and the transgene), the adenovirus vector also includes conventional control elements necessary to drive expression of the transgene in a host cell containing the transgene. Thus the vector contains a selected promoter which is linked to the transgene and located, in operable association with the transgene, between the viral sequences of the vector. Suitable promoters may be readily selected from among constitutive and inducible promoters. Selection of these and other common vector elements are conventional and many such sequences are available. *See, e.g.*, Sambrook *et al.*, and references cited therein; *see also* the general description of vector elements above.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) (*see, e.g.*, Boshart *et al.*, *Cell 41*:521-530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Inducible promoters are regulated by exogenously supplied compounds, including, the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No *et al.*, *Proc. Natl. Acad. Sci. USA, 93*: 3346-3351 (1996)), the tetracycline-repressible system (Gossen *et al.*, *Proc. Natl. Acad. Sci. USA, 89*: 5547-5551 (1992)), the tetracycline-inducible system (Gossen *et al.*, *Science 268*: 1766-1769 (1995); Harvey *et al.*, *Curr. Opin. Chem. Biol. 2*:512-518 (1998)), the RU48 pQBI-pgK-E1.1 6-inducible system (Wang *et al.*, *Nat. Biotech. 15*: 239-243 (1997); Wang *et al.*, *Gene Ther. 4*: 432-

441 (1997)) and the rapamycin-inducible system (Magari *et al.*, *J. Clin. Invest. 100*: 2865-2872 (1997)).

Other Ad Vector Elements. The vector carrying the Ad ITRs flanking the transgene and regulatory sequences (*e.g.*, promoters, polyA sequences, etc.) may be in any form which transfers these components to the host cell. Heterologous expression control elements optionally present in this vector include sequences providing signals required for efficient polyadenylation of the RNA transcript, and introns with functional splice donor and acceptor sites. Common polyadenylation (polyA) sequences which are employed in the vectors useful in this invention are derived from the papovavirus SV40 or the bovine growth hormone (BGH) gene. The polyA sequence is generally inserted 3' to, and in operable association with, the transgene sequences. A vector useful in the present invention may also contain an intron, desirably located between the promoter/enhancer sequence and the transgene, to promote mRNA stability following transcription. One possible intron sequence is also derived from SV40, and is referred to as the SV40 T intron sequence. Selection of these and other common vector elements are conventional and many such sequences are available in the art. *See, e.g.*, Sambrook *et al.*, and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27.

Co-Transfection of Adenovirus Sequences. Preferably, the replication-deficient Ad vector contains all functional adenovirus sequences required for packaging and replication upon infection of the corresponding complementing cell line of the invention. More preferably, however, a cell line of the invention which has been stably transfected to supply additional required adenovirus functions is utilized. In one embodiment, these functions may be supplied by co-transfection of an E1-complementing cell line with one or more nucleic acid molecules capable of directing expression of the required adenovirus function. As will be evident to those of skill in the art, where co-transfection is used, RCA

formation can be reduced or eliminated by minimizing overlapping homology between the Ad vector and the co-transfecting plasmid. This can be accomplished through modification of codon usage and/or the use of heterologous introns or splice sites, in either the complementing nucleic acid molecule (which supplies the essential Ad functions not supplied by either the vector or the cell) and/or modified codon usage or heterologous introns or splice sites in the Ad vector itself.

For example, a vector deleted of E1 and having a defective E2 region may be complemented in E1 complementing cell lines of the invention by transiently or stably transfecting into the cells a nucleic acid molecule (*e.g.*, a plasmid) expressing required E2 functions (*e.g.* E2a). As another example, a vector lacking E1 through E4 functions may be complemented in E1 complementing cell lines of the invention by transiently or stably transfecting the cells with a nucleic acid molecule expressing functional E2, E3 and E4 (*e.g.* E4ORF6). Construction of these nucleic acid molecules, as well as isolation of stable transfectant cell lines, is within the skill of those in the art.

Suitably, a selected recombinant vector, as described above, is introduced into E1 complementing cells from a cell line of the invention using conventional techniques, such as the transfection techniques known in the art (see, for example, Kozarsky *et al.*, *Som. Cell and Molec. Genet. 19*(5): 449-458 (1993)). Thereafter, recombinant E1-deleted adenoviruses are isolated and purified following transfection. Purification methods are well known to those of skill in the art and may be readily selected. For example, the viruses may be subjected to plaque purification and the lysates subjected to density gradient centrifugation to obtain purified virus.

Amplification of Recombinant Adenoviruses. The complementing cell lines of the invention (or derivatives thereof) may be used to amplify recombinant Ad. Suitably, the recombinant Ad will have been isolated and purified from cellular debris and other viral materials prior to use in this method. This is

particularly desirable where the rAd to be amplified is produced by methods other than those of the present invention. Suitable purification methods, *e.g.*, plaque purification, are well known to those of skill in the art.

A culture, or preferably, a suspension of cells from a complementing cell line of the invention is infected with the rAd using conventional methods. A suitable multiplicity of infection (MOI) may be readily selected. However, an MOI in the range of about 0.1 to about 100 plaque forming units (pfu) per cell, about 0.5 to about 20 pfu/cell, and/or about 1 to about 5 pfu/cell, is desirable. The cells are then cultured under conditions which permit cell growth and replication of the rAd in the presence of the Ad proteins expressed by the cell line of the invention. Suitably, the viruses are subjected to continuous passages for up to 5, 10, or 20 passages. However, where desired, the viruses may be subjected to fewer, or more passages.

The cells are subjected to two to three rounds of freeze-thawing, the resulting lysate is subjected to centrifugation to remove cellular debris, and the supernatant is collected. Conventional purification techniques such as density gradient centrifugation or column chromatography are used to concentrate the rAd, and to purify it from the cellular proteins in the lysate. Advantageously, however, the method of the invention through use of the cell lines of the invention avoids the problem of contaminating RCA which plague conventional production techniques. Verification of the absence of RCA is determined by methods described in more detail below.

Recombinant Adenovirus Particles Produced by Methods of Invention

The recombinant adenoviruses produced according to the present invention are suitable for a variety of uses and are particularly suitable for *in vivo* uses, as the present invention permits these adenoviruses to be produced in serum-free media, and in the absence of detectable RCA. Thus, the adenoviruses

produced according to the invention are substantially free of contamination with RCA.

In one embodiment, E1-deleted viruses have been deemed suitable for applications in which transient transgene expression is therapeutic (*e.g.*, p53 gene transfer in cancer, β-interferon gene transfer in cancer, PDGF gene transfer in wound healing, and vascular endothelial growth factor (VEGF) gene transfer in vascular diseases of the heart and limbs). However, the E1-deleted adenoviruses are not limited to use where transient transgene expression is desired.

Suitable doses of E1-deleted adenoviruses may be readily determined by one of skill in the art, depending upon the condition being treated, the health, age and weight of the veterinary or human patient, and other related factors. However, generally, a suitable dose may be in the range of about 10^9 to about 10^{18} virus particles per dose, preferably about 10^{10} to about 10^{17}, about 10^{11} to about 10^{16}, about 10^{12} to about 10^{15}, about 10^{13} to about 10^{14} viral particles per dose, for an adult human having weight of about 80 kg. Even more preferred doses include about 10^9, about 10^{10}, about 10^{11}, about 10^{12}, about 10^{13}, about 10^{14}, about 10^{15}, about 10^{16}, about 10^{17}, and about 10^{18} virus particles per dose for an adult human having a weight of about 80 kg. This dose may be suspended in about 0.01 mL to about 100 mL of a physiologically compatible carrier and delivered by any suitable means. Suitable delivery means include but are not limited to, by injection, either intramuscularly, subcutaneously, intravenously; by catheter infusion into, for example, the heart or lungs, or by topical application during surgery. The dose may be repeated, as needed or desired, daily, weekly, monthly, or at other selected intervals.

A Rapid, Quantitative, PCR-based Assay to Detect RCA With Increased Sensitivity (>10^{10} RCA/rAd pfu).

The present invention further provides an assay to detect replication competent viruses in production stocks of replication incompetent viruses. Specifically the present invention provides an assay to detect RCA in a

production stock of replication incompetent adenovirus. The invention further provides a method to detect replication competent viruses, particularly RCA, in production stocks of replication incompetent viruses, particularly replication incompetent adenoviruses, utilizing the assay of the present invention. Also provided are kits containing the necessary instructions and reagents to perform the assay.

The assay is based on the premise that E1-defective replication incompetent adenoviruses, such as those described herein, lack some or all of the E1 locus. The E1-encoded gene products are provided by a complementing cell line of the present invention. Therefore, in a virus preparation sufficiently purified from the host cell DNA, presence of the E1 locus is indicative of RCA. In a preferred embodiment, a PCR-based assay using a signaling probe, the signal of which is detectable and quantifiable in real time during the PCR assay, is utilized to determine the presence the E1 region in the virus preparation. In certain preferred embodiments, the assay utilizes Molecular Beacon technology (U.S. Patent No. 5,925,517) and/or TaqMan™ technology (U.S. Patent No. 5,538,848) to measure a signal generated during a PCR-based assay. U.S. Patents 5,538,848 and 5,925,517 are incorporated herein by reference in their entireties.

In general, a large-scale preparation of replication incompetent virus is subjected to DNAse treatment to eliminate or minimize host cell DNA, the virus particles are further purified by standard methods, the virus particles are lysed, and the virus genomes are subjected to PCR. For DNA viruses, standard PCR utilizing a heat stable DNA polymerase, such as Taq polymerase or Pfu polymerase. For RNA viruses such as retroviruses, RT-PCR is used, which includes a first amplification reaction with reverse transcriptase, and subsequent amplification reactions as with regular PCR. These techniques are well known to those of ordinary skill in the art, and can be found, *e.g.*, in references such as Sambrook *et al.*, *supra*.

The PCR reaction, in addition to the standard forward and reverse primers used to amplify the amplicon, also contains at least one signaling hybridization

probe for detection of the amplicon. Upon hybridization of the signaling hybridization probe to the amplicon, a detectable signal, preferably a fluorescent signal, is released, allowing measurement of the quantity of the amplicon in real time, during the course of the PCR reaction.

One suitable embodiment provides an assay for detecting the presence of replication competent virus in a production stock of replication incompetent virus comprising subjecting a sample of the virus production stock to polymerase chain reaction amplification with a forward primer and a reverse primer which amplify a region of the genome of said replication competent virus which is deleted from the genome of said replication incompetent virus, such that in the presence of said replication competent virus, a replication competent virus-specific double-stranded amplicon is formed; further allowing the sample to hybridize with a first signaling hybridization probe complementary to at least one strand of the replication competent virus-specific amplicon, where hybridization with the first signaling hybridization probe occurs under specified conditions at or below a specified detection temperature, thereby emitting a first signal, where the first signal is detectable only upon hybridization of the probe to the replication competent virus-specific amplicon, or the genome of said replication competent virus; and detecting the presence or absence of the first signal. The first signal is detectable during the PCR reaction, in real time, and the intensity of the first signal correlates with the amount of the replication competent virus-specific amplicon produced during the PCR reaction.

In this embodiment, the replication incompetent virus production stock is preferably a replication incompetent adenovirus stock, and the replication competent virus-specific amplicon is preferably amplified from a gene product which is deleted from the replication incompetent adenovirus. Suitable regions from which to amplify the replication competent virus-specific amplicon include the E1 region, the E2 region, or the E4 region (*e.g.*, E4ORF6), are described herein. Preferably the replication competent virus-specific amplicon is amplified from the E1 region.

As one of ordinary skill in the art will readily appreciate from the embodiments described above, a complementing cell line of the present invention contains a polynucleotide which encodes the gene products necessary to produce replication incompetent virus particles, and that this polynucleotide, if not sufficiently degraded by DNAse treatment as discussed above, would likely be amplified with the forward and reverse oligonucleotide primers used to produce the replication competent virus-specific amplicon. Accordingly a preferred assay of the present invention further provides a control reaction to measure the presence of host cell DNA, comprising subjecting a sample of the virus production stock to polymerase chain reaction amplification with a forward oligonucleotide primer and a reverse oligonucleotide primer which amplify a region of the host cell genome, such that in the presence of host cell DNA, a host cell-specific double-stranded amplicon is formed, further allowing the sample to hybridize with a second signaling hybridization probe complementary to at least one strand of the resulting host cell-specific double-stranded amplicon, wherein hybridization with the signaling hybridization probe occurs under specified conditions at or below a specified detection temperature, thereby emitting a second signal which is distinguishable from the first signal, discussed above, where the second signal is detectable only upon hybridization to the host cell-specific amplicon, or the host cell DNA; and detecting the presence or absence of the second signal. The second signal, like the first signal, is detectable during the PCR reaction, in real time, and the intensity of the second signal correlates with the amount of the replication competent virus-specific amplicon produced during the PCR reaction. The presence of the second signal indicates the presence of host cell DNA and thereby invalidates the assay in which the replication competent virus specific amplicon is detected.

The cell-specific amplicon can be amplified from any suitable cellular gene. A suitable cellular gene would be one that does not appear in the virus genome. Preferred genes from which to amplify the cell-specific amplicon are those which exist in multiple copies in the cellular genome, thus allowing for

greater sensitivity. Particularly preferred genes from which to amplify the cell-specific amplicon are include β-actin and GAPDH. Most preferred is to amplify the cell specific amplicon from the β-actin gene.

In a particularly preferred embodiment, amplification of the replication competent virus-specific amplicon and the cell-specific amplicon are carried out simultaneously in the same reaction tube, using a technique called multiplexing, discussed in more detail *infra*. Multiplexing is the amplification and analysis of two or more target sequences in the same PCR reaction, and is accomplished through the use of signaling hybridization probes, the signals of which are readily distinguishable by a detection instrument monitoring the reaction. A suitable detection instrument is an ABI Prism® 7700 Unit, available from PE Biosystems.

Preferred signaling hybridization probes of the present invention are Molecular Beacons (*see* U.S. Patent No 5,925,517; and Tyagi, S., and Kramer, F., *Nature Biotech. 14*:303-308 (1996), the disclosure of which is incorporated herein by reference in its entirety). Molecular Beacons are hairpin-shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid. They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule, *e.g.*, a replication competent virus-specific amplicon of the present invention. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Since the quencher moiety is a non-fluorescent chromophore and emits the energy that it receives from the fluorophore as heat, the probe is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the Molecular Beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes

the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence which can be detected by standard methods known to those of skill in the art.

Thus, in a preferred embodiment, the detection assay of the present invention utilizes a first, and preferably also a second, signaling hybridization probe, also referred to herein as a first Molecular Beacon and a second Molecular Beacon, each comprising a single-stranded polynucleotide complementary to at least one strand of the replication competent virus-specific amplicon, having a 5' terminus and a 3' terminus, with a pair of oligonucleotide arms flanking the complementary polynucleotide, consisting of a 5' arm sequence covalently linked to said 5' terminus and a 3' arm sequence covalently linked to said 3' terminus, where the pair of oligonucleotide arms form a stem duplex having a melting temperature above a specified detection temperature under specified assay conditions, but below the melting temperature of a duplex formed between the signaling hybridization probe and the complementary region of its respective amplicon (*i.e.* either the replication competent virus-specific amplicon or the cell-specific amplicon); and on each probe, a interacting label pair, comprising a fluorescent molecule conjugated to the 5' arm sequence, and a quenching molecule conjugated to the 3'arm sequence which, upon the formation of a stem duplex between said 5' arm and said 3' arm, is in sufficiently close proximity to quench the signal from the fluorescent molecule, wherein under the assay conditions at the detection temperature and in the presence of its respective amplicon, hybridization of the probe sequence to the amplicon occurs in preference to the formation of said stem duplex, sufficiently separating the fluorescent molecule from said quenching molecule, thereby allowing a fluorescent signal to be detected.

Suitable assay conditions and detection temperatures are determined based on the melting temperatures, nucleotide compositions, and lengths of the various primers and probes, as will be appreciated by one of ordinary skill in the art. One example of a set of suitable conditions is disclosed in Example 5, *infra*.

In preferred embodiments, the first signaling hybridization probe and the second signaling hybridization probe comprise distinct fluorescent molecules attached to their 5' ends, allowing two distinct fluorescent signals to be detected in a single reaction tube.

A wide variety of fluorescent molecules are suitable for use in the present invention. Suitable molecules include, but are not limited to 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxyfluorescein (TET), 2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE), hexachloro-6-carboxyfluorescein (HEX), 5-carboxyfluorescein (5-FAM), 6-carboxyrhodamine (R110), N, N'- Diethyl-2',7'-dimethyl-6-carboxyrhodamine (R6G), NED, 6-carboxytetramethylrhodamine (TAMRA), 6-carboxyrhodamine (ROX), VIC,4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY FL), [5-[(3,5-diphenyl-2H-pyrrol-2-ylidene-κN)methyl]-1H-pyrrole-2-propanoato-κN1] difluoroborate hydrogen (BODIPY 530/550), [N-[1-[4-[[3-[(2,3-dihydro -2-oxo-1H-benzimidazol-4-yl)oxy]-2-hydroxypropyl]amino]-4-methylcyclohexyl] -1-methylethyl]-5-[[5-(4-methoxyphenyl)-2H-pyrrol-2-ylidene-κN]methyl] -2,4-dimethyl -1H-pyrrole-3-propanamidato-κN1]difluoroboron (BODIPY TMR),difluoro[5- 5-(2-thienyl)-2H-pyrrol-2-ylidene-κN]methyl]-1H-pyrrole-2-propanoato-κN1]-borate hydrogen (BODIPY 558/568), difluoro[5-[[5-[(1*E*)-2-phenylethenyl] -2H-pyrrol-2-ylidene-κN]methyl]-1H-pyrrole-2-propanoato-κN1]borate hydrogen (BODIPY 564/570), difluoro[5-[[5-(1H-pyrrol-2-yl)-2H-pyrrol-2-ylidene-κN] methyl]-1H-pyrrole-2-propanoato-κN1]borate hydrogen (BODIPY 576/589), difluoro[5-[[5-[(1*E*,3*E*)-4-phenyl-1,3-butadienyl]-2H-pyrrol-2-ylidene-κN]methyl]-1H-pyrrole-2-propanoato-kN1]borate hydrogen (BODIPY 581/591), difluoro[6-[[[4-[2-[2- [[5-(2-thienyl)-1H-pyrrol-2-yl-κN]methylene]-2H-pyrrol-5-yl-κN]ethenyl]phenoxy] acetyl]amino]hexanoato]borate hydrogen (BODIPY 630/650), 1,3,6-Pyrenetrisulfonic acid, 8-[2-[[2-[(chloroacetyl)amino]ethyl]amino]-2-oxoethoxy]-, trisodium salt (Cascade Blue, N-[4-[[4-(diethylamino)phenyl] [4-(ethylamino)-1-naphthalenyl] methylene]- 2,5-cyclohexadien-1-ylidene]-N-ethylethanaminium molybdatetungstatephosphate

(Cascade Blue), 2-[5-[1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1,3-pentadienyl]-1-ethyl- 3,3-dimethyl-5-sulfo-3H-indolium inner salt (Cy5), 5-[(2-aminoethyl)amino]-1-naphthalenesulfonic acid (Edans), 7-nitrobenz-2-oxa-1,3-diazole (NBD), 2',7'-difluorofluorescein (Oregon Green 488), and Sulforhodamine 101 sulfonyl chloride (Texas Red). Preferred fluorescent molecules include 6-carboxyfluorescein (6-FAM), and VIC.

Similarly, a wide variety of quencher molecules are suitable for use in the present invention. Note that a particular quencher molecule can quench a variety of different fluorescent molecules. Therefore, a single quencher molecule can be selected to incorporate into the first and second signaling probes. Suitable quencher molecules include, but are not limited to (4-(4'-dimethylaminophenylazo)benzoic acid) succinimidyl ester (DABCYL), 4-(dimethylamine)azo benzene sulfonic acid (DABSYL), 1-dimethoxytrityloxy-3-[O-(N-4'-sulfonyl-4-(dimethylamino)-azobenzene)-3-aminopropyl]-propyl-2-O-succinoyl-long chain alkylamino-CPG (DABSYL-CPG), 9-(2-(4-carboxypiperidine-1-sulfonyl)-3,6-dimethyl-3,6-diphenyl)xanthylium (QSY™), 6-carboxytetramethylrhodamine (TAMRA), and TAMRA-NHS-Ester.

Preferred quencher molecules include DABCYL and TAMRA.

Fluorescent molecules and quencher molecules are available commercially in forms which can be attached to an existing probe by straightforward chemical protocols known to those of ordinary skill in the art, and often supplied by the manufacturer. Alternatively, certain of these molecules are available covalently linked to nucleotides which can be incorporated directly into an oligonucleotide probe during its synthesis.

Construction of suitable Molecular Beacon probes utilizes concepts which are well understood by those of ordinary skill in the art. The Molecular Beacon probe region should be 15 to 33 nucleotides long, with a melting temperature that is 7-10° C higher than the PCR annealing temperature. The melting temperature of the probe-target hybrid can be predicted using the percent GC rule, where for

an oligonucleotide of about 50 or fewer bases, T_m=4(G+C)+2(A+T), where G+C is the total number of G and C nucleotides in the chain, and A+T is the total number of A and T nucleotides in the chain. The prediction should be made for the probe sequence alone before adding the arm sequences. After selecting a probe sequence, two complementary arm sequences should be added, one on each side of the probe sequence. The stem sequences should not be complementary to the target sequence. This stem region of the Molecular Beacon should be 5 to 7 bp long, with the GC content at 70-80%. The length, sequence and GC content of the stem should be chosen such that the melting temperature is 7-10 °C higher than the annealing temperature of the PCR primers. A 5 bp stem will melt at 55-60 °C, a 6 bp stem at 60-65 °C, and a 7 bp stem at 65-70 °C. Since the G nucleotide may act as a quencher, it is best to avoid designing a Molecular Beacon with a G directly adjacent to the fluorescent dye (typically at the 5' end of the stem sequence).

The sequence of the Molecular Beacon and forward and reverse PCR primers should be designed so that there are no regions of complementarity, which may cause the Molecular Beacon to bind to primers and increase background. It is important to design the Molecular Beacon in an area where there is minimal secondary structure formation of the target. This will help prevent the template from preferentially annealing to itself faster than to the Molecular Beacon. It is recommended to design the Molecular Beacon such that it binds at or near the center of the amplicon. The distance between the 3'-end of the upstream primer and the 5'-end of the Molecular Beacon (stem) should be greater than 6 nucleotides.

Although for the PCR reactions described above Molecular Beacons are the preferred choice for the first and second signaling probes, other signaling probes may be used. For example, TaqMan™ probes, described in more detail below, are a suitable choice for the first and/or second signaling probes. Other suitable signaling probes include Scorpions™ primers (Whitcombe *et al.*, *Nature Biotech. 17*:804-807 (1999)), and the primers or probes described in Lee *et al.*,

Biotechniques 27:342-349 (1999); and Brownie *et al.*, *Nucleic Acids Research 25*:3235-3241 (1997). Molecular Beacon probes are preferred over TaqMan™ probes because they allow the use of larger amplicons, and more flexibility with the reaction conditions. Although TaqMan probes allow single copy sensitivity of amplicon detection they are limited to amplicons of less than approximately 150 bp. Molecular Beacons, while achieving the same level of sensitivity and efficiency of amplification, do not suffer from this size limitation.

In a detection assay of the present invention, forward and reverse PCR primers are chosen to produce as large a replication competent virus-specific amplicon as possible. First, the larger amplicon size will increase the probability that DNAse treatment of host cell DNA will sufficiently remove contaminating cellular DNA in the purified batches of replication incompetent viruses, because the cellular DNA (which will contain a copy of the complementation regions required for propagation of the replication incompetent virus particles, and thus would result in false positives if not sufficiently removed) will only need to be degraded to a size smaller than the amplicon size. Second, the larger replication competent virus-specific amplicon will decrease the chance of obtaining false-positive results which are the result of those recombination events between the replication incompetent virus vector DNA and the complementation region in the complementing cell which do not lead to the generation of replication competent virus (*e.g.*, a recombination of only minor parts of the complementation region). It is possible and likely that recombination could generate viruses containing only a portion of the cellular complementing DNA, yet these viruses would not be replication competent. If one targets only a small portion of the replication competent virus-specific genes in the assay, and this small region appears in this type of recombinant, replication-incompetent virus, one would score this as a replication competent virus, which would be a false positive.

On the other hand, a Molecular Beacon is an internal probe which must compete with the opposite strand of the amplicon for binding to its complementary target. Therefore, having a shorter amplicon allows the

Molecular Beacon to compete more efficiently for binding to its target and, therefore, produces better results during Molecular Beacon PCR experiments. One of ordinary skill in the art will understand the need to balance the advantages of a larger amplicon in an assay according to the present invention, with the advantages of a smaller amplicon to produce optimal results. Choice of an optimal amplicon size requires only routine experimentation, and is well within the capabilities of one of ordinary skill in the art.

Suitable sizes for amplicons used in an assay of the present invention range from about 50 base pairs in length to about 2000 base pairs in length. Preferable amplicons are about 100 bp, about 150 bp, about 200 bp, about 300 bp, about 400 bp, about 500 bp, about 600 bp, about 700 bp, about 800 bp about 900 bp, about 1000 bp, about 1250 bp, about 1500 bp, about 1750 bp or about 2000 bp in length. More preferable amplicons are about 1000 to about 1500 bp in length.

Accordingly, a series of Molecular Beacon/primer sets of increasing size are designed and the set with the largest sized amplicon that retains acceptable sensitivity is chosen for use in the detection assay. In the case of detection of RCA, the ideal first Molecular Beacon/primer set would be one that amplifies the entire E1a/E1b coding region present in the complementing cell line, and maintains sensitivity. This Molecular Beacon/primer combination would yield the lowest possible number of false-positive results in this PCR-based RCA assay.

In a particularly preferred embodiment, an RCA-specific amplicon is amplified from RCA or complementing host cell DNA with forward oligonucleotide primer 5'-GGTTTCTATGCCAAACCTTGT-3' (SEQ ID NO:12) and reverse oligonucleotide primer 5'-AACATCACTGAGGAGCAGTTCT-3' (SEQ ID NO:13). The first signaling hybridization probe/Molecular Beacon has the sequence 5'- GCAGCGAAGAAACCCATCTGAGCGGGCTGC-3' (SEQ ID NO:14). The fluorescent molecule covalently linked to the 5' end of SEQ ID NO:14 is 6-carboxyfluorescein (6-FAM), and the quencher covalently linked to

the 3' end of SEQ ID NO:14 is 4-(dimethylamine)azo benzene sulfonic acid (DABSYL). A preferred cell-specific β-actin amplicon is amplified from a complementing host cell DNA with oligonucleotide primers, and detected with a second signaling hybridization probe/Molecular Beacon purchased from Stratagene, Catalog No. 200570.

Reaction conditions for a PCR assay of the present invention include conventional PCR conditions and reagents which must be optimized for any particular set of oligonucleotide primers and probes, as one of ordinary skill in the art will readily understand. Suitable reagents are commercially available. An annealing temperature should be chosen at which the Molecular Beacon will bind efficiently to its complementary target, if present, and at which the Molecular Beacon will adopt a stem-loop conformation if it is not bound to target.

One particularly preferred detection assay of the present invention provides relative quantitation of replication competent virus vs. total virus present in the replication incompetent virus production stock. A suitable assay allows the detection of one (1) replication competent virus per 10^9 or more replication incompetent virus. Preferably, the assay will detect 1 replication competent virus per 10^{10} or more replication incompetent virus. More preferably, the assay will detect 1 replication competent virus per 10^{11}, 10^{12}, or more replication incompetent virus. Most preferably the assay will detect 1 replication competent virus per 10^{13} or more replication incompetent virus.

In order to provide relative quantitation, this particularly preferred detection assay provides measurement of the amount of replication competent virus in a virus production stock of replication incompetent virus relative to a measurement of the amount of total virus present in the virus production stock. The results are plotted as the number of replication competent virus particles present in the virus production stock relative to the number of total virus particles present in the virus production stock.

Accordingly, this particularly preferred embodiment, which incorporates the detection assays provided above, further provides subjecting a sample of the

virus production stock to polymerase chain reaction amplification with a forward oligonucleotide primer and a reverse oligonucleotide primer which amplify a region of the virus genome which is common to both replication competent and replication incompetent viruses, such that a virus-specific double-stranded amplicon is formed; allowing the sample to hybridize with a third signaling hybridization probe complementary to at least one strand of the virus-specific amplicon, where hybridization with said third signaling hybridization probe occurs under specified conditions at or below a specified detection temperature, thereby emitting a third signal, and where the third signal is detectable only upon hybridization to the virus-specific amplicon, or a virus genome; and detecting the presence or absence of the third signal.

Because the relative quantity of virus-specific amplicons in the virus production stock will be much higher than the quantities of either replication competent virus-specific amplicons, or cell-specific amplicons, this third PCR reaction is carried out separately from the PCR reactions discussed above. The reaction may be carried out with a Molecular Beacon probe similar to those discussed in detail above, or it may be carried out using a TaqMan™ probe (described in U.S. Patent No. 5,538,848, which is incorporated herein by reference in its entirety). The PCR reaction is carried out using a nucleic acid polymerase having 5' to 3' nuclease activity, and the third signaling hybridization probe comprises a fluorescent molecule and a quencher molecule which quenches the fluorescence of the fluorescent molecule in a linear, single-stranded conformation. Upon hybridization of the third signaling hybridization probe with the virus-specific amplicon, the nucleic acid polymerase digests the third signaling hybridization probe, thereby separating the fluorescent molecule from the quencher molecule, allowing detection.

The virus specific amplicon contemplated in this embodiment may be amplified from any suitable portion of the virus genome which is common to both the replication incompetent virus, and the replication competent virus. In the case of E1-deleted replication incompetent adenovirus, the virus specific amplicon

may be amplified from any adenovirus genomic region except E1. Suitable regions include, but are not limited to the E4 region, the E2 regions, the pIX region, and any of the L1-L5 regions. The E3 region may also be used, but this is less preferred, since the E3 region is deleted from many replication incompetent adenoviruses. A preferred region from which to amplify a virus-specific amplicon is the E4 region.

The following examples are provided to illustrate the production of an E1 complementing cell line of the invention and its use in producing E1-deleted adenovirus which are free of detectable RCA. These examples do not limit the scope of the invention. One skilled in the art will appreciate that although specific reagents and conditions are outlined in the following examples, modifications can be made which are meant to be encompassed by the spirit and scope of the invention.

Examples

Example 1

(A) Construction of an E1 Complementation Element and Vector pQBI-pgk-E1.1

pQBI-pgk-E1.1 is a shuttle plasmid which contains a nucleotide sequence capable of complementing E1-deleted adenovirus vectors. It contains a complementation element encoding all of the Ad5 E1a and E1b proteins including the 8.3 kDa E1b protein. The complementation element is designed such that it comprises a non-naturally occurring nucleotide sequence in its 3' most end (the region from nucleotides 3309 to 3609). This region is modified such that it encodes wild-type E1b but does not contain sufficient homology with the corresponding wild-type sequences in an Ad vector to allow for homologous recombination. In this way, a cell line transfected with such a complementation element and used to replicate E1-deleted Ad would not be expected to generate

RCA even if the Ad vector contained wild-type sequences in the region spanning nucleotides 3309 to 3609. The modifications made to the E1 sequences are shown in Figure 2 as the italicized bases below the wild-type sequence and the nucleotide sequence used to replace the wild-type sequence from nt 3309 to 3614 is shown immediately below as SEQ ID NO:1.

SEQ ID NO:1

```
GTCTTCGATA TGACGATGAA GATCTGGAAA GTCCTCCGCT ATGACGAAAC GCGGACGCGC    60
TGTCGCCCTT GTGAATGCGG GGGCAAGCAC ATCCGCAATC AACCCGTCAT GCTCGACGTC   120
ACGGAAGAAC TCCGCCCTGA CCATCTCGTC CTCGCTTGTA CGCGGGCCGA ATTCGGGTCC   180
TCCGACGAGG ACACCGACTG AGTAAGTTTA GTCTTTTTGT CTTTTATTTC AGGTCCCGGA   240
TCCGGTGGTG GTGCAAATCA AAGAACTGCT CCTCAGTGAT GTTGCCTTTA CTTCTAGCAA   300
CCCCCCCCCC CCTGAGC                                                  317
```

Nucleotides 1 to 201 of SEQ ID NO:1 are the final 67 codons (including a stop codon) of the novel sequence encoding Ad5 E1b 55 kDa protein (bold). The polypeptide encoded by nucleotides 1 to 201 is designated as SEQ ID NO:2.

Nucleotide 202 is the splice donor for the 22S E1b RNA of Ad5 (underlined).

Nucleotides 203 to 296 of SEQ ID NO:1 are the modified SV40 late region intron.

Nucleotide 297 is the splice acceptor for the 22S E1b RNA of Ad5 (underlined).

Nucleotides 298 to 315 of SEQ ID NO:1 are the final 6 codons (including a stop codon) of the novel sequence encoding Ad5 E1b 8.3 kDa protein (bold). The polypeptide encoded by nucleotides 298 to 315 is designated SEQ ID NO:3.

Plasmid pSL1180-E1 which contains the entire E1 loci of Ad5 was kindly provided by Dr. J. Wilson of the University of Pennsylvania. The silent mutations shown in Figure 2 between 3309 and 3327 are introduced to pSL1180-E1 by first generating a nucleic acid fragment comprising the mutations through the use of the polymerase chain reaction (PCR) and the two oligonucleotide primers set out below:

HC#40 5'-ATGAATGTTGTACAGGTGGC-3' (SEQ ID NO:4); and
HC#41 5'-TGACAGATCTTCATCGTCATATCGAAGACCCCGTTCAGGTTCACCTT-3' (SEQ ID NO:5).

The resulting PCR product of about 1 kb is digested with BglII and BsrGI and cloned back into the BglII-BsrGI sites of pSL1180-E1. This results in a plasmid designated "pSL1180-E1-BglII". Six separate synthetic oligonucleotides, containing the additional silent mutations and the SV40 intron shown in Figure 2, are annealed, filled in with the Klenow fragment of DNA polymerase I, and ligated by the method described in *J. Virol.* 70:4646 (1996), which is incorporated herein by reference. These six oligonucleotides are as follows:
HC#42, 5'-ATGAAGATCTGGAAAGTCCTCCGCTATGACGAAA CGCGGACGCGCTGTCGCCCTTGTGAATGCGGGGGCA-3' (SEQ ID NO:6);

HC#43, 5'-TCAGGGCGGAGTTCTTCCGTGACGTCGAGCATGACGGGT TGATTGCGGATGTGCTTGCCCCCGCATTCAC-3' (SEQ ID NO:7);

HC#44, 5'-AGAACTCCGCCCTGACCATCTCGTCCTCGCTTGTACGCGGGC CGAATTCGGGTCCTCCGACGAGGACACC -3' (SEQ ID NO:8);

HC#45, 5'-ACCACCGGATCCGGGACCTGAAATAAAAGACAAAAAGAC TAAACTTACCTCAGTCGGTGTCCTCGTCGGA-3' (SEQ ID NO:9);

HC#46, 5'-CCCGGATCCGGTGGTGGTGCAAATCAAAGAACTGCTCCT CAGTGATGTTGCCTTTACTTCTAGCAACCCC-3' (SEQ ID NO:10); and

HC#47, 5'-CGTCGTCGACTCAGGGGGGGGGGGTTGCTAGAAGT-3'(SEQ ID NO:11).

The ligated oligonucleotides are digested with BglII and SalI and ligated into the BglII and SalI sites of pSL1180-E1-BglII to create a plasmid designated "pSL1180-E1-BglII-SalI." pSL1180-E1-BglII-SalI is digested with BstBI (Klenow filled-in) and SpeI to isolate the modified E1 complementation element which comprises the modified sequence shown in SEQ ID NO:1. The fragment is ligated to the ApaI (Klenow filled in) and XbaI sites of pQBI-pgk-dl (derived from pQBI-pgk, Quantum Biotechnologies, Inc.) to create pQBI-pgk-E1.1 which operably associates the PGK promoter to the modified E1 fragment. The pQBI-pgk-E1.1 plasmid is sequenced to verify the presence of the introduced modifications.

(B) Construction of an E1 Complementation Element and Vector pQBI-pgk-E1.2

pQBI-pgk-E1.2 is another shuttle plasmid which contains a nucleotide sequence capable of complementing E1-deleted adenovirus vectors. It also contains a complementation element encoding all of the Ad5 E1a and E1b proteins including the 8.3 kDa E1b protein. The complementation element is designed such that it comprises a non-naturally occurring nucleotide sequence in its 3' most end (the region from nucleotides 3510 to 3609). In this way, a cell line transfected with such a complementation element and used to replicate E1-deleted Ad would not be expected to generate RCA even if the Ad vector contained wildtype sequences in the region spanning nucleotides 3510 to 3609. The modifications made to the E1 sequences in pQBI-pgk-E1.2 are shown diagrammatically as the boxed nucleotide substitutions in Figure 2 and the nucleotide sequence used to replace the wild-type sequence from nt 3309 to 3614 is shown immediately below as SEQ ID NO:19.

SEQ ID NO:19

```
GTGTTTGACA TGACCATGAA GATCTGGAAG GTGCTGAGGT ACGATGAGAC CCGCACCAGG   60
TGCAGACCCT GCGAGTGTGG CGGTAAACAT ATTAGGAACC AGCCTGTGAT GCTGGATGTG  120
ACCGAGGAGC TGAGGCCCGA TCACTTGGTG CTGGCCTGCA CCCGCGCTGA GTTTGGCTCT  180
AGCGATGAAG ATACAGATTG AGTAAGTTTA GTCTTTTTGT CTTTTATTTC AGGTCCCGGA  240
TCCGGTGGTG GTGCAAATCA AAGAACTGCT CCTCAGTGAT GTTGCCTTTA CTTCTAGCAA  300
CCCCCCCCCC CCTGAGC                                                  317
```

Nucleotides 1 to 201 of SEQ ID NO:19 are the final 67 codons (including a stop codon) of the naturally-occurring sequence encoding Ad5 E1b 55 kDa protein (double underlined). The polypeptide encoded by nucleotides 1 to 201 of SEQ ID NO:19 is represented as SEQ ID NO:2.

Nucleotide 202 of SEQ ID NO:19 is the splice donor for the 22S E1b RNA of Ad5 (single underlined).

Nucleotides 203 to 296 of SEQ ID NO:19 are the modified SV40 late region intron.

Nucleotide 297 of SEQ ID NO:19 is the splice acceptor for the 22S E1b RNA of Ad5 (single underlined).

Nucleotides 298 to 315 of SEQ ID NO:19 are the final 6 codons (including a stop codon) of the novel sequence encoding Ad5 E1b 8.3 kDa protein (bold). The polypeptide encoded by nucleotides 298 to 315 of SEQ ID NO:19 is represented as SEQ ID NO:3.

Plasmid pQBI-pgk-E1.2 is prepared from starting plasmid pSL1180-E1using standard techniques similar to those used in (A). The pQBI-pgk-E1.2 plasmid is sequenced to verify the presence of the introduced modifications.

(C) Construction of an E1 Complementation Element and Vector pQBI-pgk-E1.3

pQBI-pgk-E1.3 is a shuttle plasmid which contains a nucleotide sequence capable of complementing E1-deleted adenovirus vectors. It contains a complementation element encoding all of the E1a and E1b proteins including the 8.3 kDa E1b protein. The complementation element is designed such that it comprises a non-naturally occurring nucleotide sequence in its 3' most end (the

region from nucleotides 3309 to 3609). This region has been modified similarly to pQBI-pgk-E1.1, except that that it contains fewer nucleotide substitutions (shown underlined in Figure 2), which are spaced out at about one substitution every 15 nucleotides. The modifications made to the E1 sequences in pQBI-pgk-E1.3 are shown diagrammatically in Figure 2 and the nucleotide sequence used to replace the wild-type sequence from nt 3309 to 3614 is shown immediately below as SEQ ID NO:18.

SEQ ID NO:18

```
GTCTTTGACA TGACGATGAA GATCTGGAAA GTGCTGAGGT ACGACGAGAC CCGCACCAGC   60
TGCAGACCCT GCGAATGTGG CGGTAAACAC ATTAGGAACC AGCCCGTGAT GCTGGATGTC  120
ACCGAGGAGC TGAGCCCCGA TCACTTGGTC CTGGCCTGCA CCCGGGCTGA GTTTGGCTCC  180
AGCGATGAAG ATACCGATTG AGTAAGTTTA GTCTTTTTGT CTTTTATTTC AGGTCCCGGA  240
TCCGGTGGTG GTGCAAATCA AAGAACTGCT CCTCAGTGAT GTTGCCTTTA CTTCTAGCAG  300
CCGCCGCCCC CATGAGC                                                 317
```

Nucleotides 1 to 201 of SEQ ID NO:18 are the final 67 codons (including a stop codon) of the novel sequence encoding Ad5 E1b 55 kDa protein (bold). The polypeptide encoded by nucleotides 1 to 201 of SEQ ID NO:18 is represented by SEQ ID NO:2.

Nucleotide 202 is the splice donor for the 22S E1b RNA of Ad5 (underlined).

Nucleotides 203 to 296 of SEQ ID NO:1 are the modified SV40 late region intron.

Nucleotide 297 is the splice acceptor for the 22S E1b RNA of Ad5 (underlined).

Nucleotides 298 to 315 of SEQ ID NO:1 are the final 6 codons (including a stop codon) of the novel sequence encoding Ad5 E1b 8.3 kDa protein (bold). The polypeptide encoded by nucleotides 298 to 315 is represented by SEQ ID NO:3.

Plasmid pQBI-pgk-E1.3 is prepared from starting plasmid pSL1180-E1using standard techniques similar to those used in Example 1(A). The pQBI-

pgk-E1.3 plasmid is sequenced to verify the presence of the introduced modifications.

Example 2

Functional test of pQBI-pgk-E1.1 pQBI-pgk-E1.2, and pQBI-pgk-E1.3 plasmids for E1 expression

To determine whether plasmids pQBI-pgk-E1.1, pQBI-pgk-E1.2, and pQBI-pgk-E1.3, produced as described in Example 7, could complement E1-deleted adenovirus replication, monolayers of HeLa cells in 6-well plates were transiently-transfected with the plasmid DNAs using Lipofectamine (available from Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. An E1-deleted recombinant adenovirus containing a green fluorescent protein (GFP) -expression cassette in its E1-region was then added to the cells (0.1 pfu/cell). The next day the monolayers were washed extensively to remove un-adsorbed virus particles. At 48 hours post-transfection/infection the cells were harvested, extracts was made, and the presence of E1-deleted adenovirus was detected by infecting 293 cells (the standard E1-complementing cell line) with serial dilutions of the crude HeLa cell extracts. At 24 hours post-infection, the 293 cells were examined for the expression of GFP, which would be synthesized by the viruses produced in the HeLa cells only if the transiently transfected plasmid constructs expressed functional E1 gene products. The extent of E1-deleted Ad replication in this assay is directly proportional to the number of GFP expressing 293 cells. This can be directly compared between cells transfected with the complementation plasmids pQBI-pgk-E1.1, pQBI-pgk-E1.2, and pQBI-pgk-E1.3, pQBI-pgk-dl (negative control), and a plasmid containing wild-type Ad5 E1 as positive control.

The cells transiently transfected with pQBI-pgk-E1.1 yielded approximately $2x10^5$ transducing units from approximately $7x10^5$ initially transiently transfected HeLa cells; the cells transiently transfected with pQBI-

pgk-E1.2 yielded approximately $5x10^4$ transducing units from approximately $7x10^5$ initially transiently transfected HeLa cells; and the cells transiently transfected with pQBI-pgk-E1.3 yielded approximately $9x10^3$ transducing units from approximately $7x10^5$ initially transiently transfected HeLa cells. This result indicates that each of the three plasmids were complementing E1-deleted adenovirus as expected. The plasmid with the greatest number of silent nucleotide substitutions, *i.e.*, pQBI-pgk-E1.1, appeared to be the most efficient at complementing the E1-deleted adenovirus vector.

Example 3
Generation of Stable E1-Complementing Cell Lines

The target cell lines WI-38 and MRC-5 were transfected with plasmid pQBI-pgk-E1.1 to establish a permanent stably-transfected E1-complementing cell line as follows. The cells were grown on 6-cm dishes and co-transfected with 10 μg of pQBI-pgk-E1.1 and 1 μg of pIRES1neo using standard procedures (Wang *et al.*, 1995). The transfected cells were split 1:20 in fresh medium 24 hours after transfection. Following attachment of the cells to the culture plate, G418 was added to the most optimal selection concentration previously tested for WI-38 and MRC-5 cells according to standard procedures. G418 was replenished in the growth medium once every 3 ~ 4 days until sizable colonies are formed. Well-formed colonies were picked, expanded and characterized.

Each cell clone is examined for the expression of E1 gene products using Western blot analysis. Separately, each clone is tested for its ability to support the growth of an E1-deleted Ad recombinant as described above. This functional virus-complementation assay is performed in 24-well plates to test the cell clones shortly after their availability. The clones that produce the highest titer of E1 deleted adenovirus are further characterized. This characterization includes Southern blotting of chromosomal DNA to obtain an estimate of transgene copy number and a detailed restriction analysis to characterize the nature of the inserted DNA (*e.g.*, concatameric, multiple insert, etc.).

To determine the levels of RCA, if any, produced by passaging of E1-deleted Ad in the E1 complementing cell lines, standard RCA assays are performed as described (*J. Virol.* 1996, 70: 8459). Briefly, preparations of E1-deleted adenoviruses are used to inoculate non-complementing cells (A549 cells). The infected cells are cultured for approximately 14 days. In the absence of CPE, extracts are prepared from the inoculated cells and passaged onto fresh cells. Culturing of cells is continued for an additional 14 days. Evidence for growth of adenoviruses in these cultures is assessed by visual inspection, looking for viral plaques, or more general viral-induced CPE in the cultures. This type of biological amplification assay has a sensitivity of approximately 1-3 pfu of RCA in approximately 10^{10} pfu of E1-deleted Ad.

Example 4
DNase Treatment and Purification of Production Stocks of Replication Incompetent Adenovirus

Production stocks of replication incompetent adenovirus are produced in an E1-complementing cell line as described in Example 3. The cells are infected using standard virological procedures and the infection is allowed to proceed to maximum cytopathic effect. The infected cells are then subjected to two to three rounds of freeze-thawing, the resulting lysate is subjected to centrifugation to remove cellular debris, and the supernatant, containing the adenovirus particles, is collected. The virus particles are further purified by standard methods, *e.g.*, HPLC, column chromatography or density gradient centrifugation. The concentration of the production stock is estimated by plaque assay on the complementing cell line, and a sample of the production stock equivalent to about 10^{13} pfu is taken to be assayed for replication competent virus. The remainder of the production stock is frozen pending further formulation. The sample is adjusted to about 2 mM Mg^{++}, and about 50 to about 5000 units/ml of DNAse I is added to degrade non-encapsidated, contaminating cellular DNA. The DNAse digestion is incubated at about 37 °C for about 1 to about 24 hours, or until

contaminating cellular DNA is no longer amplifiable in the PCR detection assays discussed below. Following DNAse digestion, the virus particles in the sample to be tested are lysed, *e.g.*, by the use of a mild detergent.

Example 5
PCR Assay to Detect Replication Competent Adenovirus in a Production Stock of Replication Incompetent Adenovirus

The production stock of replication incompetent adenovirus is assayed for the presence of replication competent virus by measuring three parameters on the sample of virus DNA prepared as described in Example 4. The sample is estimated to contain more than 10^{13} copies of adenovirus DNA. A small aliquot (containing approximately 10^5 copies of Ad DNA) is removed to precisely measure total adenovirus DNA copies. The DNA sample is PCR amplified using forward primer 5'-ATGACACGCATACTCGGAGCT-3' (SEQ ID NO:15) and reverse primer 5'-GCCGCCCATGCAACAA-3' (SEQ ID NO:16), primers which were designed to amplify a 67-bp fragment of the Ad5 E4 region extending from nucleotide 34884 to nucleotide 34995 of the Ad5 complete genome (GenBank Accession No. M73260). A TaqMan™ PCR reaction is carried out using PCR reagent available from PE/Applied Biosystems (Part Number 402823), and according to the manufacturer's protocol. The TaqMan™ probe is designed to be specific for the Ad5 E4 gene, is added to the reaction according to the manufacturer's instructions. The probe has the sequence 5'-TGCTAACCAGCGTAGCCCCGATGT-3' (SEQ ID NO:17) and is labeled with the fluorescent molecule VIC on the 5' end and the quencher molecule 6-carboxytetramethylrhodamine (TAMRA) on the 3' end. The PCR reaction is carried out in a thermocycler capable of real-time measurement of fluorescence, *e.g.*, an ABI Prism 7700 Sequence Detection System available from PE/Applied Biosystems. The reaction is monitored for an increase in fluorescence intensity over time, and the results are correlated to a standard curve. The results are expressed as the number of viral DNA copies present in 50 ul PCR sample.

The remaining viral DNA sample is distributed into wells of 96 well plates at 1 microgram per well. The 96 well plate format of this assay lends itself to large batch size. 10^{11} copies of the adenovirus genome is approximately 4 micrograms of DNA which would require only 4 wells of the 96-well plate, while 10^{13} copies would require approximately 4 plates which is easily accommodated. Amplification and detection of both the E1 and β-actin genes are carried out in each well simultaneously using multiplex technology. Three additional wells on each 96-well plate are spiked with 1-2 copies of E1-containing DNA, and 1-2 copies of actin-containing DNA. These controls ensure that if target DNA is present in one of the test wells at a low copy number it would be detectable.

The PCR and Molecular Beacon detections are carried out using standard reagents and methods, which can be found, *e.g.*, in the Instruction Manuals provided with the Sentinel™ Molecular Beacon PCR Core Reagent Kit and Sentinel™ Molecular Beacon β-Actin Detection Kit, both available from Stratagene (Catalog Nos. 600500, and 200570, respectively, manuals available on line at http://www.stratagene.com/manuals/index.shtm) (visited April 7, 2000). The PCR primers for the E1 complementation element are forward oligonucleotide primer 5'-GGTTTCTATGCCAAACCTTGT-3' (SEQ ID NO:12) and reverse oligonucleotide primer 5'-AACATCACTGAGGAGCAGTTCT-3' (SEQ ID NO:13), which amplify a region of the Ad5 E1 complementation element extending from about nucleotide 886 in the E1a region of the complete Ad5 genome (GenBank Accession No. M73260) to within the heterologous intron derived from SV40 (*i.e.*, from about nucleotide 262 to about nucleotide 283 in SEQ ID NO:1, SEQ ID NO:18, and SEQ ID NO:19). A PCR amplification with these primers, using any of the three complementation elements produced as described in Example 1A as template, produces an amplicon of about 2.6 kb. Alternatively, both the forward and reverse primers could be suitably derived from human adenovirus sequences in the E1 region. The E1 Molecular Beacon probe has the sequence 5'-**GCAGC**GAAGAAACCCATCTGAGCGG**GCTGC**-3' (SEQ ID NO:12). The

fluorescent molecule covalently linked to the 5' end of SEQ ID NO:12 is VIC, and the quencher covalently linked to the 3' end of SEQ DI NO:12 is 4-(dimethylamine)azo benzene sulfonic acid (DABSYL). The region of the E1 Molecular Beacon probe which is complementary to the E1b gene is underlined, and this region hybridizes to a region of the Ad5 E1 gene extending from about nucleotide 2026 to about nucleotide 2045 of the complete Ad5 genome (GenBank Accession No. M73260). The portions of the 5' and 3' arms which form the hairpin in the absence of a complementary sequence are shown in bold. The Molecular Beacon for E1 detection contains VIC covalently linked to the 5' arm, and the quencher molecule DABCYL covalently linked to the 3' arm. The PCR primers Molecular Beacon for the detection of β-actin are purchased from Stratagene, and used according to the manufacturer's instructions. The β-actin probe contains the fluorescent molecule 6-carboxyfluorescein (6-FAM) covalently linked to the 5' arm and the quencher molecule DABCYL covalently linked to the 3' arm.

The amplification and detection reactions are carried out in the 96-well plate format using a thermocycler capable of simultaneous detection and measurement of fluorescence emissions at several different wave lengths, *e.g.*, an ABI PRISM 7700 unit. The reaction starts with a two-minute denaturation step at 95 °C, which is followed by about 40 cycles of amplfication, each having a 30-second denaturation step at 95 °C, a one-minute annealling step at about 50-60 °C, and a 30-second extension step at 72 °C. The thermocycler is set to detect and report fluorescence during the annealling/extension step of each cycle. The results are displayed as an amplification plot, which measures the intensity of fluorescence of each of the fluorescent molecules as a function of the number of cycles completed in the PCR reaction.

It is essential to co-amplify and measure each of these DNA sequences in the entire DNA sample in order to ensure that any signal detected for E1 DNA is in fact from a RCA rather than a very small amount of cellular DNA that might be in the final DNA preparation. For instance, 50 μg of viral DNA is tested as

described above in 50 wells of a 96 well plate and a signal for E1 DNA is detected in one of the wells, it is imperative to ascertain that the signal originated from viral rather than cellular DNA. This is accomplished by measuring a cellular gene, in this case, β-actin, in the same 50 μg (50 wells) of viral DNA.

The data collected in the TaqMan E4 PCR reaction and the Molecular Beacon amplification/detection reactions is combined to determine the number of replication competent adenovirus genomes present per 10^{13} replication incompetent adenovirus genomes. The criteria for the presence of RCA using this assay will be the lack of a signal for β-actin DNA (as an indication that all non-encapsidated DNA has been removed from the sample), and any level of detection of the targeted E1 DNA sequence. If both β-actin and E1 DNA are detected in the assay, the assay will not be valid, and the analysis will need to be performed on a new sample of the production stock of replication incompetent adenovirus, which will be subjected to additional DNAse digestion as described in Example 4.

Example 6
Hybrid Selection to Enrich for Virus DNA in Samples taken from Production Stocks of Replication Incompetent Adenovirus.

If the presence of cellular DNA poses a problem in the assay, a hybrid selection approach is used to enrich the sample for virus DNA. In order to enrich the preparation of viral DNA for the presence of DNA genomes derived from RCA, the genomes are selected for using hybrid-selection (Jagus, R. *Meth. Enzymol. 152*:567-572 (1987)). Bacterial plasmids containing the E1 gene and the β-actin gene are immobilized onto membrane filters. These filters are used to hybridize the final purified viral DNA genome sample, prepared as described in Example 4. Cellular DNA and DNA from RCA hybridizes to the E1 DNA in the plasmid on the filter, while DNA from the vast majority of the virus (E1-deleted rAd) will not hybridize and will be washed away after hybridization. Hybridized cellular DNA and DNA from RCA is eluted from the filters by heating, the eluted DNA is collected and ethanol precipitated. This cellular gene hybrid-selection, decreases the chances of obtaining false-positive results (*i.e.*, a signal for E1 that would be from cellular rather than viral DNA). In order to avoid contamination with small amounts of the sequences immobilized on the filter, the fragment of E1 DNA which is cloned into the plasmid is smaller than the amplicon used in the PCR assay. Accordingly, it does not contain the binding sites for the PCR primers, and hence is not amplified.

Example 7
Construction of E1 Complementation Elements and Vectors pQBI-pgk-E1.1, pQBI-pgk-E1.3, and pQBI-pgk-E1.3

Shuttle plasmids pQBI-pgk-E1.1, pQBI-pgk-E1.3, and pQBI-pgk-E1.3, as described above in Example 1, were constructed by the following method.

(a) Production of pQBI-pgk-E1.2.

The 5' portion of the SV40 intron as shown in Figure 2 (*i.e.*, nucleotides 202 to 243 of SEQ ID NO:1, SEQ ID NO:18, or SEQ ID NO:19) was introduced

into plasmid pSL1180-E1 as follows. A nucleic acid fragment comprising a portion of the E1 gene and part of the SV40 intron was generated through the use of the polymerase chain reaction (PCR) using pSL1180-E1 as template, and the two oligonucleotide primers set out below:

HC#40 5'-ATGAATGTTGTACAGGTGGC-3' (SEQ ID NO:4); and

HC#48 5'-CCACCGGATC CGGGACCTGA AATAAAAGAC AAAAAGACTA AACTTACCTC ATCTGTATC TTCATCG-3'(SEQ ID NO:21).

BamHI and BsrGI and cloned into the BamHI-BsrGI sites of pSL1180-E1. The resulting plasmid was designated pSL1180-E1-BamHI (#99). Plasmid pSL1180-E1-BamHI was then digested with HindIII to remove about 2.3-kb of the upstream portion of the E1 gene. The remaining plasmid was religated, in order to eliminate an upstream SalI site located at the beginning of the E1 fragment, resulting in pSL1180-E1-BamHI-dl (#104).

The portion of the SV40 intron downstream of the BamHI site and the final 6 codons (including the stop codon) of the 8kD E1b protein with silent mutations, *i.e.*, nucleotides 244 to 315 of SEQ ID NO:19, was introduced into pSL1180-E1-BamHI-dl as follows. The following complementary oligonucleotides were annealed:

HC#49 5'-GATCCGGTGG TGGTGCAAAT CAAAGAACTG CTCCTCAGTG GATGTTGCCT TTACTTCTAG CAACCCCCCC CCCCCTGAG-3' (SEQ ID NO:22), and

HC#50 5'-TCGACTCAGG GGGGGGGGGG TTGCTAGAAG TAAAGGCAAC ATCCACTGAG GAGCAGTTCT TTGATTTGCA CCACCACCG-3' (SEQ ID NO:23).

Annealing of these oligonucleotides results in a BamHI cohesive end on the 5' terminus and a SalI cohesive end on the 3' terminus. pSL1180-E1-BamHI-dl (#104) was digested by BamHI and SalI and ligated with the annealed oligonucleotides to create pSL1180-E1-BamHI-dl-SalI(#106). Sequence

analysis showed that the sequence was correct.

Plasmid pSL1180-E1-BamHI-dl-SalI(#106) was then digested with HindIII and ligated with the about 2.3-kb HindIII fragment from pSL1180-E1 to reconstruct the whole E1 fragment. This manipulation resulted in pSL1180-E1-BamHI-SalI (#109).

Plasmid pQBI-pgk-dl was derived from pQBI-pgk, available from Quantum Biotechnologies, Inc., by digestion with PstI and self-ligation to remove the Ad TPL, GFP, and Neo genes, resulting in pQBI-pgk-dl(#107).

Plasmid pQBI-pgk-dl(#107) was digested by BglII, followed by a fill-in reaction with Klenow, and was then digested with XhoI to obtain an about 570-bp fragment containing the pgk promoter. This fragment was then ligated into pSL1180-E1-BamHI-SalI(#109) digested with SpeI, filled in with Klenow and then digested with XhoI, to create pQBI-pgk-E1.2-dlpA(#116).

Plasmid pQBI-pgk-E1.2-dlpA(#116) was then digested by BsrGI and SalI to obtain a fragment of about 1377 bp containing the portion of the E1 gene PCR amplified as above, the SV40 intron, and the final 6 codons (including the stop codon) of the 8kD E1b protein with silent mutations. A second sample of pQBI-pgk-E1.2-dlpA (#116) was digested with BstBI, subjected to a fill-in reaction with Klenow, and then digested with BsrGI, to obtain the plasmid backbone fragment of about 5459 bp. Plasmid pQBI-pgk-dl(#107) was digested by XhoI and PvuII to obtain the BGH poly-adenylation sequence. These three fragments were ligated to create pQBI-pgk-E1.2(#121), the final construct of E1.2. The region of pQBI-pgk-E1.2 extending from about 19 nucleotides before the BglII site shown in Fig. 2, and extending to the end of the sequence shown in Fig. 2 is depicted herein as SEQ ID NO:19.

(b) Production of pQBI-pgk-1.1.

A nucleic acid molecule which incorporates the silent mutations extending from about nucleotide 20 to about nucleotide 243 of SEQ ID NO:1 was created by annealing three overlapping sets of complementary nucleotides. The oligonucleotide pairs are as follows:

HC#52 5'-GATCTGGAAA GTCCTCCGCT ATGACGAAAC GCGGACGCGC TGTCGCCCTT GTGAATGCGG GGGCAAGCAC ATCCG-3' (SEQ ID NO:25), and

HC#53 5'-GATTGCGGAT GTGCTTGCCC CCGCATTCAC AAGGGCGACA GCGCGTCCGC GTTTCGTCAT AGCGGAGGAC TTTCCA-3' (SEQ ID NO:26);

HC#54 5'-CAATCAACCC GTCATGCTCG ACGTCACGGA AGAACTCCGC CCTGACCATC TCGTCCTCGC TTGTACGCGG GCCGA-3' (SEQ ID NO:27), and

HC#55 5'-CGAATTCGGC CCGCGTACAA GCGAGGACGA GATGGTCAGG GCGGAGTTCT TCCGTGACGT CGAGCATGAC GGGTT-3' (SEQ ID NO:28); and

HC#56 5'-ATTCGGGTCC TCCGACGAGG ACACCGACTG AGGTAAGTTT AGTCTTTTTG TCTTTTATTT CAGGTCCCG-3' (SEQ ID NO:29), and

HC#57 5'-GATCCGGGAC CTGAAATAAA AGACAAAAAG ACTAAACTTA CCTCAGTCGG TGTCCTCGTC GGAGGACC-3' (SEQ ID NO:30).

The annealed nucleic acid molecule was ligated into plasmid pQBI-pgk-E1.2(#121) which had been digested with BglII and BamHI, to create pQBI-pgk-E1.1-oligo(#122).

The silent mutations shown in the first 18 nucleotides of Figure 2 (*i.e*, nucleotides 1 to 18 of SEQ ID NO:1 were introduced into pQBI-pgk-E1.1-oligo(#122) by generating a nucleic acid fragment comprising the mutations through the use of the polymerase chain reaction (PCR) using pSL1180-E1 as template, and the two oligonucleotide primers set out below:

HC#40 5'-ATGAATGTTGTACAGGTGGC-3' (SEQ ID NO:4); and

HC#51 5'-TGACAGATCT TCATCGTCAT ATCGAAGACC CCGTTCAGGT TCACCTT-3' (SEQ ID NO:24, BglII site underlined).

The resulting PCR product of about 1 kb was digested with BglII and BsrGI and cloned into the BglII-BsrGI sites of pQBI-pgk-E1.1-oligo(#122). The

resulting plasmid was designated pQBI-pgk-E1.1(#123), the final construct of E1.1. The region of pQBI-pgk-E1.1 extending from about 19 nucleotides before the BglII site shown in Fig. 2, and extending to the end of the sequence shown in Fig. 2, is depicted herein as SEQ ID NO:1.

(c) Production of pQBI-pgk-E1.3.

The single underlined silent mutation shown in the first 18 nucleotides of Figure 2, *i.e*, at nucleotide 15 of SEQ ID NO:18, was introduced into pQBI-pgk-E1.1-oligo(#122) by generating a nucleic acid fragment comprising the mutation through the use of the polymerase chain reaction (PCR) using pSL1180-E1 as template, and the two oligonucleotide primers set out below:

HC#40 5'-ATGAATGTTGTACAGGTGGC-3' (SEQ ID NO:4); and

HC#58 5'-TTCCAGATCT TCATCGTCAT GTCAAAGACC CCGTTCAGGT TCACCTT-3' (SEQ ID NO:31, BglII site underlined).

The resulting PCR product of about 1 kb was digested with BglII and BsrGI and cloned into the BglII-BsrGI sites of pQBI-pgk-E1.1-oligo(#122). The resulting plasmid was designated pQBI-pgk-E1.3-PCR(#125).

A nucleic acid molecule which incorporates the silent mutations extending from about nucleotide 20 to about nucleotide 278 of SEQ ID NO:18 was created by annealing four overlapping sets of complementary nucleotides. The oligonucleotide pairs are as follows:

HC#59 5'-GATCTGGAAA GTGCTGAGGT ACGACGAGAC CCGCACCAGC TGCAGACCCT GCGAATGTGG CGGTAAACAC ATTAGGAACC AGCCC-3' (SEQ ID NO:32), and

HC#60 5'-CATCACGGGC TGGTTCCTAA TGTGTTTACC GCCACATTCG CAGGGTCTGC AGCTGGTGCG GGTCTCGTCG TACCTCAGCA CTTTCCA-3' (SEQ ID NO:33);

HC#61 5'-GTGATGCTGG ATGTCACCGA GGAGCTGAGC CCCGATCACT TGGTCCTGGC CTGCACCCGG GCTGAGTTTG GCTCCAGCGA TGAAG-3' (SEQ ID NO:34), and

HC#62 5'-CGGTATCTTC ATCGCTGGAG CCAAACTCAG

CCCGGGTGCA GGCCAGGACC AAGTGATCGG GGCTCAGCTC CTCGGTGACA TCCAG-3' (SEQ ID NO:35);

HC#63 5'-ATACCGATTG AGGTAAGTTT AGTCTTTTTG TCTTTTATTT CAGGTCCCGG ATCCGGTGGT GGTGCAAATC AAAGAACTGC TCCTC-3' (SEQ ID NO:36), and

HC#64 5'-TCCACTGAGG AGCAGTTCTT TGATTTGCAC CACCACCGGA TCCGGGACCT GAAATAAAAG ACAAAAAGAC TAAACTTACC TCAAT-3'(SEQ ID NO:37); and

HC#65 5'-AGTGGATGTTGCCTTTACTTCTAGCAGCCGCCGCCCC ATGAGTCGAGCATGCATCTAGAGGGCC-3'(SEQ ID NO:38), and

HC#66 5'-CTCTAGATGCATGCTCGACTCATGGGGGCGGCGCTGCTA GAAGTAAAGGCAACA-3' (SEQ ID NO:39).

The annealed nucleic acid molecule was ligated into pQBI-pgk-E1.3-PCR(#125) which had been digested with BglII and ApaI, to create pQBI-pgk-E1.3(#127), the final construct of E1.3. The region of pQBI-pgk-E1.3 extending from about 19 nucleotides before the BglII site shown in Fig. 2, and extending to the end of the sequence shown in Fig. 2, is depicted herein as SEQ ID NO:18.

All three versions of E1 construct have been sequenced to verify the the PCR-amplified and oligo modified regions.

***

All publications cited in this specification are hereby incorporated herein by reference. While the invention has been described with reference to a particularly preferred embodiment, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. An isolated nucleic acid molecule comprising a polynucleotide which encodes at least 5 contiguous amino acids of a naturally occurring adenovirus polypeptide, wherein the sequence of said polynucleotide is not a naturally-occurring adenovirus nucleotide sequence.

2. The nucleic acid molecule of claim 1, wherein said adenovirus polypeptide is essential for adenovirus replication.

3. The nucleic acid molecule of claim 1, wherein said polynucleotide encodes at least 5 contiguous amino acids of an E1 polypeptide.

4. The nucleic acid molecule of claim 3, wherein said polynucleotide encodes at least the 5 terminal amino acids of the about 8.3 kDa E1b polypeptide.

5. The nucleic acid molecule of claim 3, wherein the sequence of said polynucleotide is selected from the group consisting of:

(a) nucleotides 1 to 198 of SEQ ID NO:1;

(b) nucleotides 298 to 312 of SEQ ID NO:1;

(c) nucleotides 1 to 315 of SEQ ID NO:1;

(d) nucleotides 1 to 198 of SEQ ID NO:18;

(e) nucleotides 298 to 312 of SEQ ID NO:18;

(f) nucleotides 1 to 315 of SEQ ID NO:18;

(g) nucleotides 298 to 312 of SEQ ID NO:19; and

(h) nucleotides 1 to 315 of SEQ ID NO:19.

6. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (a).

7. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (b).

8. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (c).

9. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (d).

10. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (e).

11. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (f).

12. The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (g).

13 The nucleic acid molecule of claim 5, wherein the sequence of said polynucleotide (h).

14. The nucleic acid molecule of claim 1, wherein the sequence of said polynucleotide is less than 97%, but greater than about 60% identical to a naturally occurring adenovirus nucleotide sequence.

15. The nucleic acid molecule of claim 15, wherein the sequence of said polynucleotide is less than 90% identical to a naturally occurring Ad5 nucleotide sequence.

16. The nucleic acid molecule of claim 14, wherein the sequence of said polynucleotide is less than 97%, but greater than about 60% identical to nucleotides 1 to 198 of SEQ ID NO:20.

17. The nucleic acid molecule of claim 14, wherein the sequence of said polynucleotide is less than 97%, but greater than about 60% identical to nucleotides 287 to 301 of SEQ ID NO:20.

18. The nucleic acid molecule of claim 16, wherein the sequence of said polynucleotide is less than 90% identical to nucleotides 1 to 198 of SEQ ID NO:20.

19. The nucleic acid molecule of claim 17, wherein the sequence of said polynucleotide is less than 90% identical to nucleotides 287 to 301 of SEQ ID NO:20.

20. The nucleic acid molecule of claim 1, wherein said polynucleotide will not hybridize under stringent conditions to a naturally occurring adenovirus polynucleotide.

21. The nucleic acid molecule of claim 20, wherein said naturally occurring adenovirus polynucleotide is SEQ ID NO:20.

22. A complementation element comprising the nucleic acid molecule of claim 1.

23. The complementation element of claim 22, further comprising a promoter.

24. The complementation element of claim 22, wherein said nucleic acid molecule comprises all the protein coding regions of an adenovirus E1 locus.

25. A complementation element comprising the nucleic acid molecule of claim 1 which encodes an essential adenovirus protein selected from the group consisting of an E1 protein, an E2 protein, and an E4 protein.

26. A cell capable of complementing the replication of replication-incompetent adenovirus, comprising the complementation element of claim 22.

27. A vector comprising the nucleic acid molecule of claim 1.

28. A method of making a host cell comprising stably transforming or transfecting a cell with the vector of claim 27.

29. A system for producing adenovirus vectors, comprising:
the cell of claim 26; and
a replication incompetent adenovirus vector.

30. An assay for detecting the presence of replication competent virus in a production stock of replication incompetent virus comprising:

(a) subjecting a sample of said production stock to polymerase chain reaction amplification with a forward oligonucleotide primer and a reverse oligonucleotide primer which amplify a region of the genome of said replication competent virus which is deleted from the genome of said replication incompetent virus, such that in the presence of said replication competent virus, a replication competent virus-specific double-stranded amplicon is formed;

(b) allowing said sample to hybridize with a first signaling hybridization probe complementary to at least one strand of said replication competent virus-specific amplicon, wherein hybridization with said first signaling

hybridization probe occurs under specified conditions at or below a specified detection temperature, thereby emitting a first signal, and wherein said first signal is detectable only upon hybridization of said first signaling hybridization probe to said replication competent virus-specific amplicon, or the genome of said replication competent virus; and

(c) detecting the presence or absence of said first signal.

31. The assay of claim 30, wherein said first signaling hybridization probe comprises:

(a) a single-stranded polynucleotide complementary to at least one strand of said replication competent virus-specific amplicon, having a 5' terminus and a 3' terminus;

(b) flanking the complementary polynucleotide, a pair of oligonucleotide arms consisting of a 5' arm sequence covalently linked to said 5' terminus and a 3' arm sequence covalently linked to said 3' terminus, said pair of oligonucleotide arms forming a stem duplex, said stem duplex having a melting temperature above said detection temperature under said assay conditions, but below the melting temperature of a duplex formed between said first signaling hybridization probe and the complementary region of said replication competent virus-specific amplicon; and

(c) a first interacting label pair, comprising a first fluorescent molecule conjugated to the 5' arm sequence, and a first quenching molecule conjugated to the 3'arm sequence which, upon the formation of a stem duplex between said 5' arm and said 3' arm, is in sufficiently close proximity to quench the signal from said first fluorescent molecule, wherein under the assay conditions at the detection temperature and in the presence of said replication competent virus-specific amplicon, hybridization of the probe sequence to the replication competent virus-specific amplicon occurs in preference to the formation of said stem duplex, sufficiently separating said first fluorescent

molecule from said first quenching molecule, thereby allowing a first fluorescent signal to be detected.

32. The assay of claim 31, wherein said first fluorescent molecule is selected from the group consisting of 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxyfluorescein (TET), 2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE), hexachloro-6-carboxyfluorescein (HEX), 5-carboxyfluorescein (5-FAM), 6-carboxyrhodamine (R110), N, N'- Diethyl-2',7'-dimethyl-6-carboxyrhodamine (R6G), NED, 6-carboxytetramethylrhodamine (TAMRA), 6-carboxyrhodamine (ROX), and VIC.

33. The assay of claim 32, wherein said first fluorescent molecule is 6-carboxyfluorescein (6-FAM).

34. The assay of claim 31, wherein said first quencher molecule is selected from the group consisting of (4-(4'-dimethylaminophenylazo)benzoic acid) succinimidyl ester (DABCYL), 4-(dimethylamine)azo benzene sulfonic acid (DABSYL), 1-dimethoxytrityloxy-3-[O-(N-4'-sulfonyl-4-(dimethylamino)-azobenzene)-3-aminopropyl] -propyl-2-O-succinoyl-long chain alkylamino-CPG (DABSYL-CPG), 9-(2-(4-carboxypiperidine-1-sulfonyl) -3,6-dimethyl-3,6-diphenyl)xanthylium (QSY™), 6-carboxytetramethylrhodamine (TAMRA), and TAMRA-NHS-Ester.

35. The assay of claim 31, wherein said first fluorescent molecule is 6-carboxyfluorescein (6-FAM), and wherein said first quencher is (4-(4'-dimethylaminophenylazo)benzoic acid) succinimidyl ester (DABCYL).

36. The assay of claim 30, wherein said replication competent virus-specific amplicon comprises at least 150 base pairs.

37. The assay of claim 36, wherein said replication competent virus-specific amplicon comprises at least 1000 base pairs.

38. The assay of claim 37, wherein said replication competent virus-specific amplicon comprises at least 1500 base pairs.

39. The assay of claim 30, which detects replication competent virus at a sensitivity of 1 replication competent virus particle per 1 X 10^9 replication incompetent virus particles.

40. The assay of claim 39, which detects replication competent virus at a sensitivity of 1 replication competent virus particle per 1 X 10^{11} replication incompetent virus particles.

41. The assay of claim 40, which detects replication competent virus at a sensitivity of 1 replication competent virus particle per 1 X 10^{13} replication incompetent virus particles.

42. The assay of claim 30, wherein said replication competent virus is a replication competent adenovirus, and wherein said replication incompetent virus is a replication incompetent adenovirus.

43. The assay of claim 42, wherein said replication incompetent adenovirus comprises a deletion in the E1 region, and wherein said replication competent virus-specific amplicon is amplified from the E1 region of said replication competent adenovirus.

44. The assay of claim 43, wherein said replication competent virus-specific amplicon comprises an adenovirus E1 region.

45. The assay of claim 30, further comprising

(a) subjecting a sample of said production stock to polymerase chain reaction amplification with a forward oligonucleotide primer and a reverse oligonucleotide primer which amplify a region of the host cell genome, such that in the presence of host cell DNA, a host cell-specific double-stranded amplicon is formed;

(b) allowing said sample to hybridize with a second signaling hybridization probe complementary to at least one strand of said host cell-specific double-stranded amplicon, wherein hybridization with said signaling hybridization probe occurs under specified conditions at or below a specified detection temperature, thereby emitting a second signal which is distinguishable from said first signal, and wherein said second signal is detectable only upon hybridization to said host cell-specific amplicon, or said host cell DNA; and

(c) detecting the presence or absence of said second signal.

46. The assay of claim 45, wherein said second signaling hybridization probe comprises:

(a) a single-stranded polynucleotide complementary to at least one strand of said host cell-specific amplicon, having a 5' terminus and a 3' terminus;

(b) flanking the complementary polynucleotide, a pair of oligonucleotide arms consisting of a 5' arm sequence covalently linked to said 5' terminus and a 3' arm sequence covalently linked to said 3' terminus, said pair of oligonucleotide arms forming a stem duplex about 3-25 nucleotides in length, said stem duplex having a melting temperature above said detection temperature under said assay conditions, but below the melting temperature of a duplex formed between said second signaling hybridization probe and the complementary region of said host cell-specific amplicon; and

(c) a second interacting label pair, comprising a second fluorescent molecule conjugated to the 5' arm sequence, wherein the signal of said second fluorescent molecule is distinct from the signal of said first fluorescent molecule,

and a second quenching molecule conjugated to the 3'arm sequence which, upon the formation of a stem duplex between said 5' arm and said 3' arm, is in sufficiently close proximity to quench the signal from said second fluorescent molecule, wherein under the assay conditions at the detection temperature and in the presence of said host cell-specific amplicon, hybridization of the probe sequence to the host cell-specific amplicon occurs in preference to the formation of said stem duplex, sufficiently separating said second fluorescent molecule from said second quenching molecule, thereby allowing a second fluorescent signal to be detected.

47. The assay of claim 46, wherein said polymerase chain reaction amplifications are carried out simultaneously in a single reaction.

48. The assay of claim 46, wherein said second fluorescent molecule is selected from the group consisting of 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxyfluorescein (TET), 2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE), hexachloro-6-carboxyfluorescein (HEX), 5-carboxyfluorescein (5-FAM), 6-carboxyrhodamine (R110), N, N'- Diethyl-2',7'-dimethyl-6-carboxyrhodamine (R6G), NED, 6-carboxytetramethylrhodamine (TAMRA), 6-carboxyrhodamine (ROX), and VIC.

49. The assay of claim 48, wherein said second fluorescent molecule is VIC.

50. The assay of claim 46, wherein said second quencher is selected from the group consisting of (4-(4'-dimethylaminophenylazo)benzoic acid) succinimidyl ester (DABCYL), 4-(dimethylamine)azo benzene sulfonic acid (DABSYL), 1-dimethoxytrityloxy-3-[O-(N-4'-sulfonyl-4-(dimethylamino)-azobenzene)-3-aminopropyl] -propyl-2-O-succinoyl-long chain alkylamino-CPG (DABSYL-CPG), 9-(2-(4-carboxypiperidine-1-sulfonyl)- 3,6-dimethyl-3,6-

diphenyl)xanthylium (QSY™), 6-carboxytetramethylrhodamine (TAMRA), and TAMRA-NHS-Ester.

51. The assay of claim 50, wherein said second fluorescent molecule is VIC, and wherein said second quencher is (4-(4'-dimethylaminophenylazo)benzoic acid) succinimidyl ester (DABCYL).

52. The assay of claim 45, wherein said host cell-specific amplicon comprises at least 150 base pairs.

53. The assay of claim 52, wherein said host cell-specific amplicon comprises at least 1000 base pairs.

54. The assay of claim 53, wherein said host cell-specific amplicon comprises at least 1500 base pairs.

55. The assay of claim 45, wherein said host cell-specific amplicon amplifies a gene selected from the group consisting of β-actin and GAPDH.

56. The assay of claim 55, wherein said host cell-specific amplicon amplifies the β-actin gene.

57. The assay of claim of claim 45, further comprising:

(a) subjecting a sample of said production stock to polymerase chain reaction amplification with a forward oligonucleotide primer and a reverse oligonucleotide primer which amplify a region which is common to both replication competent and replication incompetent viruses, such that a virus-specific double-stranded amplicon is formed;

(b) allowing said sample to hybridize with a third signaling hybridization probe complementary to at least one strand of said virus-specific

amplicon, wherein hybridization with said third signaling hybridization probe occurs under specified conditions at or below a specified detection temperature, thereby emitting a third signal, and wherein said third signal is detectable only upon hybridization to said virus-specific amplicon, or a virus genome; and

(c) detecting the presence or absence of said third signal.

58. The assay of claim 57, wherein said polymerase chain reaction amplification is performed using a nucleic acid polymerase having 5' to 3' nuclease activity; wherein said third signaling hybridization probe comprises a fluorescent molecule and a quencher molecule which quenches the fluorescence of said fluorescent molecule in a linear, single-stranded conformation; and wherein, upon hybridization of said third signaling hybridization probe with said virus-specific amplicon, said nucleic acid polymerase digests said third signaling hybridization probe thereby separating said fluorescent molecule from said quencher molecule, allowing detection.

59. The assay of claim 58, wherein amplification of said virus-specific amplicon reaction is carried out separately from the amplification reactions of said replication competent virus-specific amplicon, and said cell-specific amplicon.

60. The assay of claim 58, wherein detection of the relative levels of said virus- specific amplicon and said replication competent virus-specific amplicon are measured, in real time, over the course of said assay; thereby allowing measurement of the relative quantities of replication competent virus and replication incompetent virus in said production virus stock.

61. The assay of claim 60, which detects replication competent virus at a sensitivity of 1 replication competent virus particle per 1 X 10^9 replication incompetent virus particles.

62. The assay of claim 61, which detects replication competent virus at a sensitivity of 1 replication competent virus particle per 1 X 10^{11} replication incompetent virus particles.

63. The assay of claim 62, which detects replication competent virus at a sensitivity of 1 replication competent virus particle per 1 X 10^{13} replication incompetent virus particles.

64. The assay of claim 58, wherein detection of said second signal under standard conditions indicates the presence of excess cellular DNA in said sample, thereby invalidating the assay.

65. A method to detect the presence of replication competent virus in a production stock of replication incompetent virus, comprising testing one or more samples of said virus production stock in the assay of claim 30.

66. A method to detect the presence of replication competent virus in a production stock of replication incompetent virus, comprising testing one or more samples of said virus production stock in the assay of claim 45.

67. A method to detect the presence of replication competent virus in a production stock of replication incompetent virus, comprising testing one or more samples of said virus production stock in the assay of claim 57.

68. A diagnostic kit for testing for the presence of replication competent virus in a production stock of replication incompetent virus, comprising:

(a) an manual instructing how to perform the assay 30;

(b) a forward oligonucleotide primer and a reverse oligonucleotide primer which are specific for a region of the genome of said replication competent virus and which is deleted from the genome of said replication incompetent virus, wherein said oligonucleotide primers, under specified conditions, facilitate amplification of a replication competent virus-specific amplicon; and

(c) a first signaling hybridization probe complementary to at least one strand of said replication competent virus-specific amplicon.

69. The kit of claim 68, further comprising:

(a) a forward oligonucleotide primer and a reverse oligonucleotide primer which are specific for a host cell gene, wherein said oligonucleotide primers, under specified conditions, facilitate amplification of a host cell-specific amplicon; and

(b) a second signaling hybridization probe complementary to at least one strand of said host cell-specific amplicon, wherein the signal of said second signaling hybridization probe is distinguishable from said first signaling hybridization probe.

70. The kit of claim 69, further comprising:

(a) a forward oligonucleotide primer and a reverse oligonucleotide primer which amplify a viral genomic region which is common to both replication competent and replication incompetent viruses, and which under specified conditions facilitate amplification of a virus-specific amplicon; and

(b) a third signaling hybridization probe complementary to at least one strand of said virus-specific amplicon.

71. The nucleic acid molecule of claim 21, wherein said polynucleotide comprises a heterologous intron.

72. The nucleic acid molecule of claim 71, wherein said heterologous intron is an SV40 late region intron.

73. The complementation element of claim 24, wherein said adenovirus E1 locus further comprises a heterologous intron in the E1b region.

74. The complementation element of claim 73, wherein said heterologous intron is an SV40 late region intron.

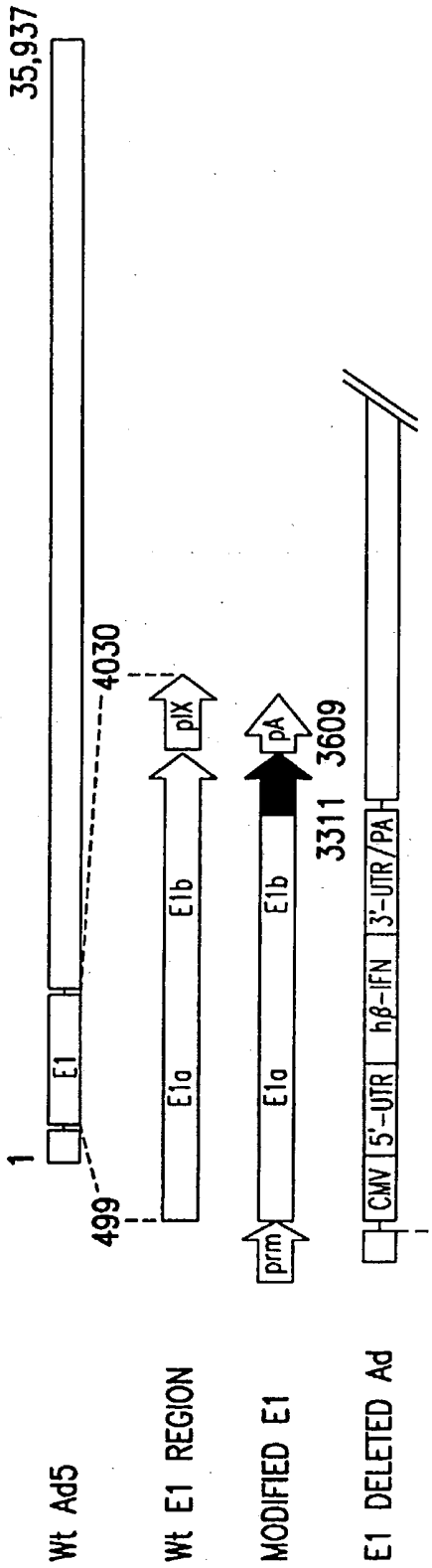
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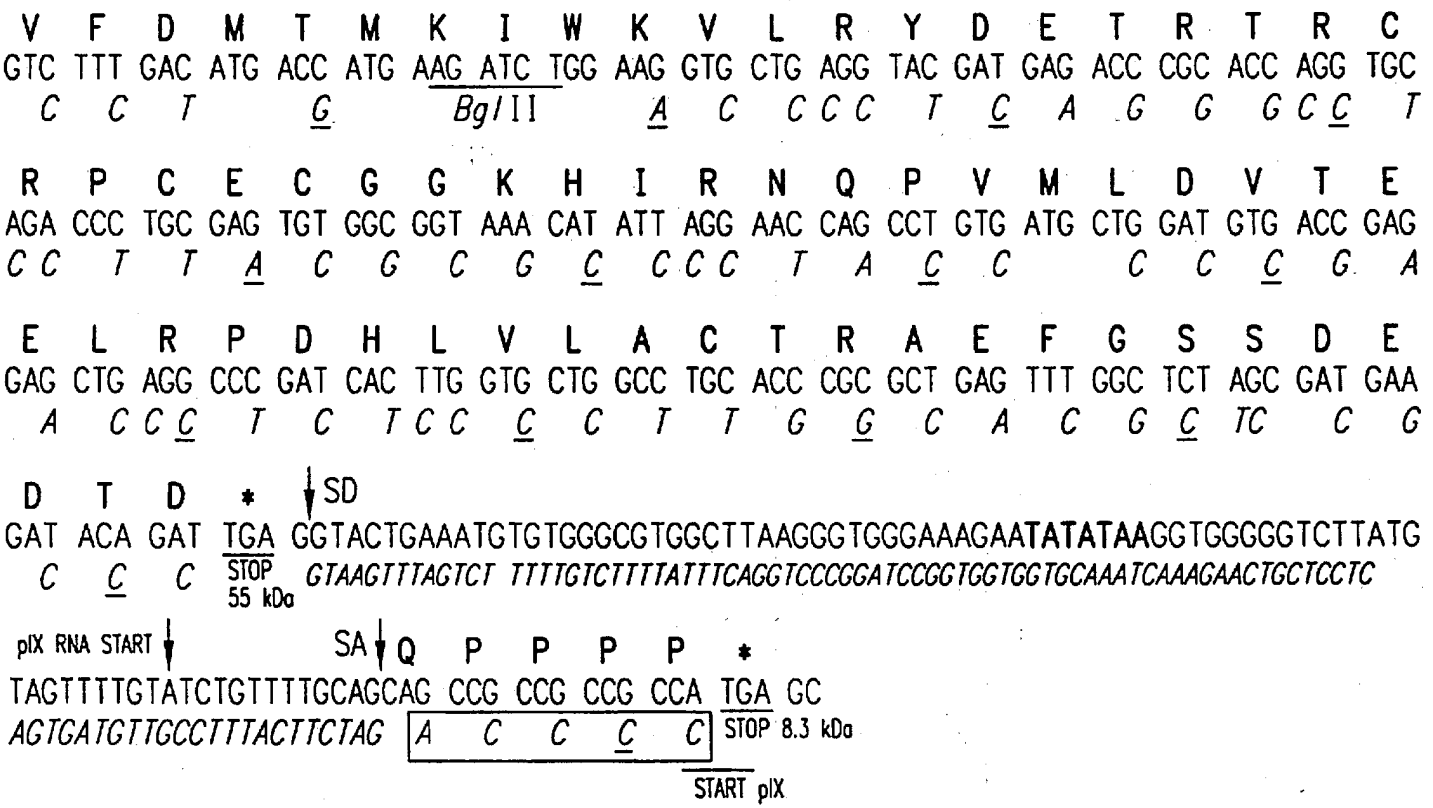
FIG.1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C C T G BglII A C C C C T C A G G G C C T
R P C E C G G K H I R N Q P V M L D V T E
AGA CCC TGC GAG TGT GGC GGT AAA CAT ATT AGG AAC CAG CCT GTG ATG CTG GAT GTG ACC GAG
C C T T A C G C G C C C C T A C C C C G A
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A C C C T C T C C C C T T G G C A C G C TC C G
D T D * SD
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pIX RNA START SA Q P P P P *
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FIG.2

SEQUENCE LISTING

<110> Genovo, Inc. *et al.*

<120> Methods and Compositions for the Manufacture of Replication Incompetent Adenovirus

<130> 1915.001PC03

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<150> US 60/196,266
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acg cgg acg cgc tgt cgc cct tgt gaa tgc ggg ggc aag cac atc cgc 96
Thr Arg Thr Arg Cys Arg Pro Cys Glu Cys Gly Gly Lys His Ile Arg
20 25 30

aat caa ccc gtc atg ctc gac gtc acg gaa gaa ctc cgc cct gac cat 144
Asn Gln Pro Val Met Leu Asp Val Thr Glu Glu Leu Arg Pro Asp His
35 40 45

ctc gtc ctc gct tgt acg cgg gcc gaa ttc ggg tcc tcc gac gag gac 192
Leu Val Leu Ala Cys Thr Arg Ala Glu Phe Gly Ser Ser Asp Glu Asp
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acc gac tga gtaagtttag tctttttgtc ttttatttca ggtcccggat 241
Thr Asp
65

ccggtggtgg tgcaaatcaa agaactgctc ctcagtgatg ttgcctttac ttctag caa 300

Gln

317

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<400> 12
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<213> SV40 Virus

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accgaggagc tgaggcccga tcacttggtg ctggcctgca cccgcgctga gtttggctct 180
agcgatgaag atacagattg aggtactgaa atgtgtgggc gtggcttaag ggtgggaaag 240
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<210> 21
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<210> 22
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<211> 85
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<223> Description of Artificial Sequence: Primer

<400> 38
agtggatgtt gcctttactt ctagcagccg ccgcccccat gagtcgagca tgcatctaga 60
gggcc 65

<210> 39
<211> 55
<212> DNA
<213> Artificial

<220>
<223> Description of Artificial Sequence: Primer

<400> 39

ctctagatgc atgctcgact catgggggcg gcggctgcta gaagtaaagg caaca 55

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— *with international search report*

(88) Date of publication of the international search report:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND COMPOSITIONS FOR THE MANUFACTURE OF REPLICATION INCOMPETENT ADENOVIRUS

(57) Abstract: The present invention relates to complementing cell lines for the production of replication incompetent viruses, which significantly reduce or eliminate the presence of replication competent viruses. Methods to make and use the complementing cell lines are also provided, as are nucleic acid molecules, polynucleotides, and vectors for making the cell lines. In particular, the present invention relates to complementing cell lines for the production of replication incompetent adenoviruses (Ad), which significantly reduce or eliminate the presence of replication competent Ad (RCA) and can serve for the large scale production of infectious replication incompetent adenovirus particles that may be used for the treatment of human patients as for example in gene therapy. The present invention further relates to an assay for detecting the presence of replication competent virus particles, in particular RCA, in a stock of infectious replication incompetent virus particles, in particular replication incompetent adenovirus particles, which employs a real time quantitative PCR assay with a sensitivity level to detect one replication competent virus particle per $\geq 10^9$ replication incompetent virus particles.

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INTERNATIONAL SEARCH REPORT

Inter. onal Application No
PCT/US 00/33123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 994 132 A (CHAMBERLAIN JEFFREY S ET AL) 30 November 1999 (1999-11-30) abstract examples ---	1-29
X	WO 97 15679 A (KELLEY WILLIAM MARK ;UNIV PENNSYLVANIA (US); WILSON JAMES M (US)) 1 May 1997 (1997-05-01) figure 9; example 2 claims ---	1-29
X	US 5 994 128 A (FALLAUX FRITS JACOBUS ET AL) 30 November 1999 (1999-11-30) the whole document --- -/--	1-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 June 2001	29/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Panzica, G

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 13499 A (CIBA GEIGY AG ;SCRIPPS RESEARCH INST (US); MEMEROW GLEN R (US); VO) 2 April 1998 (1998-04-02)	1-29
Y	abstract examples 1,2 page 112 -page 131	30-74
Y	EP 0 745 690 A (NEW YORK HEALTH RES INST) 4 December 1996 (1996-12-04) the whole document	30-74
A	WO 95 13399 A (NEW YORK HEALTH RES INST) 18 May 1995 (1995-05-18) the whole document	30-74
X	HERISSE J ET AL: "NUCLEOTIDE SEQUENCE OF ADENOVIRUS 2 DNA FRAGMENT ENCODING FOR THE CARBOXYLIC REGION OF THE FIBER PROTEIN AND THE ENTIRE E4 REGION" NUCLEIC ACIDS RESEARCH,GB,IRL PRESS LTD., OXFORD, vol. 9, no. 16, 25 August 1981 (1981-08-25), pages 4023-4042, XP002051626 ISSN: 0305-1048 the whole document	1-29

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-4 and dependents relate to an extremely large number of possible compounds. In fact, the claim contains a definition by which that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely the sequences with the identity numbers 1, 18, 19 and 20 (as reported in claims 5, a-d, and 16).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/33123

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